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Cell cycle activation in p21 dependent pathway: An alternative mechanism of organophosphate induced dopaminergic neurodegeneration

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ABSTRACT

In the previous study, we demonstrated that dichlorvos induces oxidative stress in dopaminergic neuronal cells and subsequent caspase activation mediates apoptosis. In the present study, we evaluated the effect and mechanism of dichlorvos induced oxidative stress on cell cycle activation in NGF-differentiated PC12 cells. Dichlorvos exposure resulted in oxidative DNA damage along with activation of cell cycle machinery in differentiated PC12 cells. Dichlorvos exposed cells exhibited an increased expression of p53, cyclin-D1, pRb and decreased expression of p21suggesting a re-entry of differentiated cells into the cell cycle. Cell cycle analysis of dichlorvos exposed cells revealed a reduction of cells in the G_0/G_1 phase of the cell cycle (25%), and a concomitant increase of cells in S phase (30%) and G2/M phase (43.3%) compared to control PC12 cells. Further, immunoblotting of cytochrome *c*, Bax, Bcl-2 and cleaved caspase-3 revealed that dichlorvos induces a caspase-dependent cell death in PC12 cells. These results suggest that Dichlorvos exposure has the potential to generate oxidative stress which evokes activation of cell cycle machinery leading to apoptotic cell death *via* cytochrome *c* release from mitochondria and subsequent caspase-3 activation in differentiated PC12 cells.

1. Introduction

Dichlorvos is a synthetic insecticide that belongs to the family of chemically related organophosphate (OP) pesticides. India is the world's largest OP producer, and companies make many hazardous products, including Dichlorvos [1]. Dichlorvos can be released into the environment as a major degradation product of other OP insecticides, such as trichlorfon, naled and metrifonate [18,22]. Organophosphates, including dichlorvos, have been reported to exert their primary pharmacological and toxicological effects through the inhibition of acetylcholinesterase (AChE) [3]. Studies have also reported that Dichlorvos exposure is toxic to dopaminergic (DA) neurons and this could lead to Parkinson-like symptoms in rats. In addition to this, findings from a recent study indicate that production of ROS by chronic dichlorvos treatment in rats may underlie the selective vulnerability of Dopaminergic neurons to apoptotic cell death [6,32]. Moreover, it has been established that chronic Dichlorvos exposure can induce oxidative stress, resulting in over-expression of pro-apoptotic genes, DNA damage and finally leading to caspase-dependent apoptotic cell death in rat brain [20,31].

Parkinson's disease (PD) is a neurodegenerative disease that is characterized by DA neuronal cell death and the presence of Lewy bodies in the substantial nigra pars compacta (SNpc) [25]. Epidemiological studies suggest that pesticide exposure is a significant risk factor for PD, which is the second most common neurodegenerative disorder. Some studies have linked geographical distribution of pesticide usage with the prevalence of PD. For example, case–control surveys from several countries including the United States, Canada, Australia, Hong Kong, and Taiwan have shown statistically significant associations between pesticide exposures and PD [7,8,12].

Recent experimental evidence revealed that aberrant cell cycle progression may play an important role in the pathogenesis of PD [17]. The abnormal expression of cell cycle markers and cell cycle reentry has been found in the post- mitotic neurons of the central nervous system in PD patients [4,34]. Moreover, recent findings suggest that

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neurodegeneration is associated with activation of the cell cycle machinery in post -mitotic neurons [21,27,9]. Owing to the relevance of PD with DA cell loss and the finding that Dichlorvos selectively induces DA neuronal cell death in rats, a DA neuronal cell line, PC12 was employed in this study. One of the most salient features of PC12 cell line is that its capacity to respond to nerve growth factor (NGF) by exiting the cell cycle and undergoing neuronal differentiation [15].

The exploitation of post-mitotic cells in this study is important since one of the suggested intracellular pathways implicated in neuronal death relates to the re-entry of neurons (post-mitotic cells) in the cell cycle. The differentiated PC12 cells highly resemble sympathetic neurons in phenotype; they extend branched axons, are electrically excitable and responsive to neurotransmitters, express a variety of neuronal markers and retain their capacity for dopamine synthesis, storage, uptake and release [14,15]. Therefore, we believed that NGF- differentiated PC12 cells were suitable for delineating the possible role of cell cycle activation in dichlorvos induced DA neuronal cell death.

Therefore, the present study was designed to evaluate the role of Dichlorvos induced oxidative stress on activation of cell cycle machinery and its possible role in DA neuronal cell death.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle's medium (DMEM) was purchased from Hyclone (Utah, USA). Fetal bovine serum (FBS), Horse serum and 0.25% trypsin were purchased from Invitrogen (California, USA).Tissue culture plates, 25 cm2 *T*-flasks and cell scrapers were purchased from Greiner bio-one (St. Gallen, Switzerland). 0.2 QL syringe filters were purchased from Millipore (Massachusetts, USA). H2DCFDA and dichlorvos were procured from Sigma-Aldrich (Missouri, USA). Primary antibodies were purchased from Santa Cruz Biotechnologies (California, USA), whereas secondary antibodies were purchased from Bangalore Genei (Bangalore, India). All other chemicals used in this study were of tissue culture grade.

2.2. PC12 cell culture

PC12 cells were obtained from the National Center for Cell Sciences, Pune, India. PC12 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 5% (v/v) horse serum, and a 1% (v/v) penicillin-streptomycin antibiotics mixture and were grown in an atmosphere of 95% air and 5% CO2 at 37 °C. PC12 cell neuronal differentiation was induced by administration of nerve growth factor-7S (NGF, 50 ng/mL) in Dulbecco's Modified Eagle medium (DMEM) supplemented with 1% FBS for 7 days, as already described [19,32].

3. Determination of cell viability

3.1. MTT assay

Cell viability was quantitated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. Briefly, ~2 × 104 cells/mL were treated with Dichlorvos (0–450 μM) and incubated in the presence of 0.5 mg/mL MTT for 1 h at 37 °C and then solubilized by adding a solution containing 50% dimethylformamide and 20% sodium dodecyl sulphate (pH 4.7). The amount of MTT formazan produced was determined by measuring its absorbance at a test wavelength of 570 nm and a reference wavelength of 655 nm.

3.2. LDH assay

The assay principle relies on the fact that lactate dehydrogenase can pass the plasma membrane of damaged (necrotic or apoptotic) cells. Outside the cell, lactate dehydrogenase catalyzes the oxidation of lactate to pyruvate and the reduction of NAD^+ to $NADH^+H^+$ in the same time. $NADH^+ + H^+$ reduces a yellowish tetrazolium salt resulting in a deep red formazan salt. Consequently, the absorption of the formazan correlates with lactate dehydrogenase activity. Cells were plated at a density of 1×10^5, according to manufacturer's instructions (Roche Diagnostics, Germany) and the assay was started. After incubation, cells were centrifuged at 1000 ×*g*, the supernatant was pipetted in a fresh 96 well plate, and LDH reagent was added. After half an hour, stop solution (1 N HCl) was added and absorption was measured at 420 nm with a correction wavelength of 620 nm using an ELISA microplate reader.

4. Measurement intracellular ROS production

H2DCF-DA (dihydrodichlorofluoresceindiacetate) is widely used to evaluate 'cellular oxidative stresses. After passing through the plasma membrane, this lipophilic and non-fluorescent compound is de-esterified to a hydrophilic alcohol [H2DCF (dihydrodichlorofluorescein)] that may be oxidized to fluorescent DCF (2′,7′-dichlorofluorescein) by a process usually considered to involve ROS (reactive oxygen species). PC12 cells ($1x10^6$ cells per 3 mL in six-well plates) were exposed to Dichlorvos (30 μM) for 24 h and rinsed three times with PBS. H2DCF-DA dye (20 μM) was added and cells were incubated at 37 °C for 30 min. After 30 min incubation, the cells were harvested and washed with PBS twice. The samples were analyzed using a flow cytometer (Becton Dickinson).

4.1. Quantification of 8-hydroxy-deoxyguanosine (8-OHdG)

Genomic DNA was isolated from the control and PC12 exposed cells and 8-OHdG levels were measured as an index of DNA oxidation using DNA oxidation kit (Cayman, Chemicals, USA) as per manufacturers protocol.

4.2. RNA isolation and RT-PCR of p53, CyclinD1, and Rb

RNA was extracted, and cDNA was synthesized from control and dichlorvos exposed PC12 cells.The cDNA was used for the RT-PCR analysis of the above mentioned genes. PCR amplification for amplification of GAPDH (internal control), p53, CyclinD1 and Rb cDNA products (1 μL) were subjected to semi quantitative PCR analysis on a gradient thermal cycler instrument (Eppendorf, Germany). Expression of p53, CyclinD1, Rb and GAPDH was evaluated by PCR analysis using sequence specific primers corresponding to the sequence in the open reading frame. 10 μL PCR mixtures were prepared, consisting of 1 unit of Taq polymerase, 2 μM of each primer for GAPDH, p53, CyclinD1, and RB, along with 200 μM of each dNTP.

PCR cycle of GAPDH comprised of initial denaturation at 95 °C for 2 min. The amplification was then carried out for 35 cycles consisting 1 min each at 94 °C (denaturation), 62 °C (annealing) and 72 °C (elongation). Final extension was done at 72 °C for 10 min.

PCR cycle of p53 comprised of initial denaturation at 95 °C for 1 min. The amplification was then carried out for 34 cycles consisting 94 °C for 1 min (denaturation), 30 s at 57.8 °C (annealing) and 1 min at 72 °C (elongation). Final extension was done at 72 °C for 10 min.

PCR cycle of CyclinD1 comprised of initial denaturation at 95 °C for 1 min. The amplification was then carried out for 34 cycles consisting 94 °C for 1 min (denaturation), 30 s at 56.5 °C (annealing) and 1 min at 72 °C (elongation). Final extension was done at 72 °C for 10 min.

PCR cycle of Rb comprised of initial denaturation at 95 °C for 3 min. The amplification was then carried out for 32 cycles consisting 94 °C for 1 min (denaturation), 30 s at 57 °C (annealing) and 1 min at 72 °C (elongation). Final extension was done at 72 °C at 10 min.

GAPDH was used as internal control for densitometry analysis of the PCR products using Image J software from the NIH (Bethesda, MA, USA).to compare the relative mRNA expression of various genes from

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