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Inhibitory effects of pesticides on proteasome activity: Implication in Parkinson's disease

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Epidemiological studies have suggested a correlation of pesticides and Parkinson's disease (PD) while genetic and biochemical studies have implicated the ubiquitin–proteasome system (UPS) in the pathogenesis of PD. In the present studies, we tested the hypothesis that pesticide exposure increases the risk of developing PD by inhibiting the UPS. The effects of pesticides on proteasome activity were examined in SK-N-MC neuroblastoma cells overexpressing a GFP-conjugated proteasome degradation signal, GFP^{u}. Six out of 25 representative pesticides, including rotenone, ziram, diethyldithiocarbamate, endosulfan, benomyl, and dieldrin, showed inhibitory effects on proteasome activities at low concentrations (10 nM to 10 μM). Unlike proteasome inhibitors, they did not inhibit 20 S proteasome activities in cell lysates. Except for rotenone, the other five pesticides did not induce significantly cellular oxidative stress. The cytotoxic effects of these pesticides were closely correlated with proteasome inhibition. Our results suggest proteasome inhibition as a potential mechanism for the epidemiological association of pesticides and PD.

Keywords: Parkinson's disease; Ubiquitin–proteasome system; Pesticides; Environmental toxin; Neurotoxicity; Oxidative stress; Cell death; Degron; Rotenone; Ziram

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by relatively selective degeneration of dopaminergic neurons in the substantia nigra. Epidemiological studies indicate that pesticides are the leading candidates of environmental toxins that may contribute to the pathogenesis of PD (Baldi et al., 2003; Betarbet et al., 2000; Di Monte, 2003; Di Monte et al., 2002; Gorell et al., 1998; Jimenez-Jimenez et al., 1992; Semchuk et al., 1992; Tanner et al., 1989). Rotenone, a common pesticide and an inhibitor of mitochondrial complex I, has been used to develop a PD model, with pathological features of degeneration of dopaminergic neurons and formation of cytoplasmic inclusions (Betarbet et al., 2000). Other pesticides, such as paraquat, dieldrin, and maneb, have been reported to cause degeneration of dopaminergic neurons (McCormack et al., 2002; Meco et al., 1994; Sanchez-Ramos et al., 1998; Uversky, 2004). The mechanisms, however, remain largely unknown.

Although the majority of PD patients have sporadic onset and do not have a familial history, genetic findings have provided important insights into pathogenic mechanisms of PD (Allam et al., 2005; Greenamyre et al., 2003; Tsang and Soong, 2003). Many forms of familial PD cases have been found to be caused by mutations in components related to the ubiquitin–proteasome system (UPS), highlighting the potential importance of UPS in PD (Greenamyre and Hastings, 2004; McNaught and Olanow, 2003). For example, mutations in parkin, an E3 ubiquitin ligase, have been found in juvenile-onset Parkinsonism (Kitada et al., 1998) and result in loss of ubiquitin ligase activity (Shimura et al., 2000). A point mutation in ubiquitin C-terminal hydrolase L1 (UCH-L1), a neuronal-specific deubiquitinating enzyme, can lead to reduced catalytic activity of the enzyme and PD (Leroy et al., 1998). Alterations in α-synuclein gene cause autosomal dominant PD (Chartier-Harlin et al., 2004; Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004). α-Synuclein is a major component of the cytoplasmic inclusions found in PD brains (Lewy bodies) (Spillantini et al., 1997), and also a potential substrate of UPS (Bennett et al., 1999; Liani et al., 2004; Nonaka et al., 2005). These familial cases have led researchers to consider that acquired proteasome dysfunction may be involved in the pathogenesis of sporadic PD. Consistent with this hypothesis, decreased proteasome activity in substantia nigra of PD brains has been described (McNaught and Jenner, 2001). In addition, systemic exposure to proteasome inhibitors in rats causes motor dysfunction, loss of dopaminergic neurons, and formation of inclusions resembling Lewy bodies (McNaught et

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al., 2004). All these findings are suggestive of the critical role of UPS dysfunction in PD.

Given that exposure to some toxins and pesticides is reported to be associated with PD and acquired proteasome abnormality may contribute to PD pathogenesis, we hypothesized that some pesticides may lead to UPS inhibition. Indeed, it was recently reported that maneb inhibits proteasome activity (Zhou et al., 2004). However, no extensive and systematic study on pesticides and proteasome function has been reported to date. In the present study, we used an efficient cellular model that utilizes a 26 S proteasome reporter system to examine a series of representative pesticides for their effects on proteasome activities. For the pesticides that show proteasome inhibitory effects, we further examined their direct action on proteasome activity in cell lysates and their effects on cellular oxidative stress. The relationship between cytotoxicity and proteasome inhibition was also investigated.

Materials and methods

Proteasome inhibitors, clasto-lactacystin-β-lactone, epoxomicin, MG132, were purchased from Sigma (St. Louis, MO). Pesticides were obtained from Chemservice (West Chester, PA). GFP^u reporter construct was a generous gift from Dr. Ron Kopito.

Cell cultures and pesticide treatment

Transfected or non-transfected SK-N-MC neuroblastoma cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin under humidified atmosphere of 5% CO_2. The pesticides were freshly prepared. Proteasome inhibitors and most pesticides were dissolved in dimethyl sulfoxide (DMSO), except for 2,4-D-dimethylamine, diethyldithiocarbamate, methamidophos, and paraquat in H_2O. DMSO concentration is equal or below 0.1% in culture medium. The cell cultures were treated with 25 pesticides at the concentrations ranging from 10 nM to 10 μM. Culture media were changed every other day.

Determination of cellular 26 S ubiquitin-dependent proteasome activity

SK-N-MC cells were transfected with the reporter plasmid GFP^u, developed by Bence et al. (2001). The reporter gene consists of a short degron CL1 that is ligated to the COOH-terminus of GFP. CL1, encoding a fragment of amino acids ACKNWFSSLSHFVIHL, was shown to be a degradation signal for ubiquitin–proteasome system (Gilon et al., 1998). A clonal cell line stably expressing GFP^u was isolated and designated SK-N-MC^u. The product of GFP^u was continuously degraded and kept at very low levels under normal conditions. Compromised proteasome function reduces the clearance capacity by UPS and increase steady-state GFP^u levels. Cellular fluorescence was measured by a flow cytometer (Beckman Coulter) at 495 nm excitation and 525 nm emission wavelength. The cultured cells were trypsinized for 5 min and suspended as individual cells before flow cytometry analysis.

Determination of 20 S proteasome activity in cell lysate

The non-transfected SK-N-MC cells were washed with PBS and lysed by freeze thawing in H_2O containing 1 mM dithiothreitol (DTT). The cell lysate was then centrifuged and the supernatant was taken. Protein concentration was measured by Lowry method. Assay buffer includes 25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.03% SDS in a final volume of 200 μl. Proteasome inhibitors or pesticides were pre-incubated with cell lysate for 30 min at room temperature. Assay starts with addition of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC, 100 μM) that measures chymotryptic proteasome activity. The rate of fluorescence increment was monitored by a fluorimetric multiplate reader (PerkinElmer Wallac) and calculated from the linear portions of the curves (10 to 30 min).

Assays of cell death and cell viability

Total attached cells, including live and dead cells, were harvested by trypsinization and counted on a hemacytometer. Propidium iodide (PI) was added to the culture medium (1 μg/ml) 30 min before trypsinization. Trypsinized cells were analyzed by flow cytometer at 620 nm emission, along with GFP analysis. The percentage of dead cells was obtained from the PI-positive cells in total. The live cell number = total attached cell number × (1 − percent of dead cells).

Detection of intracellular oxidative stress

Production of intracellular reactive oxygen species (ROS) was monitored by oxidation of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (C-H_2DCFDA, Molecular Probes). Cleavage of the ester groups by intracellular esterases and oxidation by ROS generate intracellular dichlorofluorescein (DCF), which are highly fluorescent. After loading C-H_2DCFDA, the cultures were treated with proteasome inhibitors or pesticides for 2 h. The cells were then harvested by trypsinization and analyzed by flow cytometry at 525 nm.

Statistical analysis

The data are presented as mean ± SEM. Statistical analysis for comparison of mean values was performed by one-way ANOVA followed by Dunnett's post test. $P < 0.05$ was considered as statistically significant.

Results

Ubiquitin-dependent proteasome activity in cell cultures (validation of method)

Transfected SK-N-MC^u cells were used for quantitation of cellular ubiquitin-dependent proteasome activity. The sensitivity and reproducibility of this assay were tested using three proteasome inhibitors: lactacystin, epoxomicin and MG132. The inhibitors, at a series of concentrations, were incubated with SK-N-MC^u cells for 24 h. The cellular fluorescence intensity represents the levels of accumulated GFP^u products. Each inhibitor induced a dose-dependent increase and a peak value of fluorescence intensity with a maximal fluorescence intensity for lactacystin being 17.5 ± 2.7 (mean ± SEM) at a concentration of 5 μM, 15.9 ± 1.3 at 20 nM of epoxomicin, and 16.8 ± 0.4 at 1.5 μM of MG132 (Figs. 1A, B). Higher concentrations of inhibitors beyond these values resulted in lower cellular fluorescence, presumably owing to cell death and