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Proteasome subunit and opioid receptor gene expression down-regulation induced by paraquat and maneb in human neuroblastoma SH-SY5Y cells

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ABSTRACT

Paraquat (PQ) and maneb (MB) are able to induce neurotoxic effects by promoting α-synuclein (α-syn) aggregates and altering tyrosine hydroxylase (TH), thus increasing the risk of Parkinson's disease (PD). These pesticides promote neurotoxic effects also by affecting proteasome function that normally regulate protein turnover. We investigated the effects of the two pesticides exposure on multiple targets involved in PD, using SH-SY5Y cells. First, we evaluated TH and α-syn protein levels following PQ and MB cell exposure and a significant increase of these protein levels was observed. Subsequently, since a relationship between ubiquitin/proteasome and opioid receptors has been proposed, the effects of pesticides on their gene expression have been investigated. A decrease of β1 and Rpt3 proteasome subunit mRNA levels, together with the μ and δ opioid receptor down-regulation, was detected.

The reported alterations, here simultaneously observed, help to clarify the involvement of multiple biological markers implicated in PD, often separately evaluated.

1. Introduction

Parkinson's disease (PD) is a movement disorder characterized by dopaminergic cells degeneration of *substantia nigra* (SN) *pars compacta*. Its pathogenetic mechanisms are likely triggered by abnormal accumulation of proteins, mitochondrial dysfunction and oxidative stress (Jellinger, 2012). The intraneuronal cytoplasmic aggregates, termed "Lewy Bodies", induce neuronal toxicity involving an impaired clearance of α-synuclein (α-syn) protein, likely caused by the ubiquitin–proteasome 26 S system (UPS) dysfunction (Xilouri et al., 2013).

The 26 S proteasome is an evolutionary conserved biochemical complex which works to modulate several protein levels. Its structure consists of the 20 S core particle (CP) which catalyzes peptide bond hydrolysis, and the 19 S regulatory particle (RP) involved in the recruitment, binding and folding of substrates allowing them to enter in the 20 S CP (Tomko and Hochstrasser, 2013). The 20 S CP is organized in four heteroheptameric rings; the inner rings consist of seven distinct β-subunits (β1–β7), whit the β1, β2, and β5 containing the proteolytic active sites; the outer rings consist of seven different α-subunits (α1–α7). The 19 S RP is organized in two different sections: the base and the lid. The base contains nine subunits: six RP triphosphatases (Rpt1–6), and three RP non-ATPases (Rpn1, 2, and 13); the lid comprises nine different Rpn subunits (Rpn3, 5–9, 11, 12, and Rpn15).

Proteasome activity represents a crucial step in the maintenance of cellular dynamics and its dysfunction is involved in neurodegenerative disorders associated with protein accumulation and inclusion development, such as in PD (Dennissen et al., 2012). It has been showed that proteasome activity is decreased in dopaminergic neurons of the SN in PD subjects and that a loss of 20 S proteasome α subunits also occurs (McNaught and Jenner, 2001; McNaught et al., 2002).

Several environmental factors have been proposed to increase the risk of PD development; epidemiological studies suggest a significant role played by pesticides, including paraquat (PQ) and maneb (MB) as risk factors in the aging-related diseases (Costello et al., 2009).

Abbreviations: PQ, paraquat; MB, maneb; α-syn, α-synuclein; TH, tyrosine hydroxylase; PD, Parkinson's disease; SN, substantia nigra; UPS, ubiquitin–proteasome 26 S system; CP, core particle; RP, regulatory particle; Rpt, regulatory particle triphosphateses; Rpn, regulatory particle non-ATPases; MOP, μ-opioid receptor; DOP, δ-opioid receptor.

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PQ (1,1′-dimethyl-4,4′-bipyridinium-dichloride) is a highly toxic quaternary nitrogen herbicide that induces neuronal toxicity mainly by oxidative stress and apoptotic-related mechanisms (Franco et al., 2010). The effects of PQ seem selective for the SN dopaminergic cells (McCormack et al., 2002) and this pesticide also induces α-syn aggregation probably caused by proteasome dysfunction (Yang and Tiffany-Castiglioni, 2007).

MB (Mn-containing ethylene-bis-dithiocarbamate) is a fungicide used in agriculture; similarly to PQ, MB induces oxidative stress, inhibition of proteasomal activity and pre-synaptic α-syn aggregation in dopaminergic cell cultures (Zhou et al., 2004).

Furthermore, a possible role for the opioid system has been suggested in the PD pathophysiology (Marti et al., 2005, 2010) and a relationship between proteasome complex and opioid receptors has been reported. Proteasome inhibitors attenuate the agonist-induced decrease of opioid receptor levels and, in the absence of agonists, increase the polyubiquitinated levels of μ (MOP) and δ (DOP) opioid receptors (Chaturvedi et al., 2001).

Based on these premises, the main aim of the study was to investigate the effects of PQ and MB, alone or in combination, on molecular targets involved in PD development in human neuroblastoma SH-SY5Y cells.

As a first step, we evaluated the effect of 48 h pesticides exposure on tyrosine hydroxylase (TH) and α-syn protein levels. In fact, TH alterations in PD are well known and the α-syn aggregates are a typical pathognomonic feature of this neurodegenerative pathology (Zhu et al., 2012). In the same experimental condition, the gene expression levels of selected proteasome subunits involved in the proteolysis machinery were investigated. To this purpose, mRNA levels for Rpn9, Rpt3 and β1 subunits, respectively located in the lid, base and 20 S CP sections, were analyzed; finally, MOP and DOP gene expression alterations were also examined.

This approach could clarify whether the different alterations of investigated targets may occur simultaneously, thus helping to understand the PD/pesticides relationships.

2. Material and methods

2.1. Cell culture

Human SH-SY5Y neuroblastoma cells, purchased from ICLC-IST (Genoa, Italy), were used, cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For all experiments, cells were allowed to reach 80% confluence before treatments. Cells were exposed to specific concentrations of PQ or MB (Sigma–Aldrich, Milan, Italy) or their combinations.

2.2. MTT cell viability assay

Cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were plated at 37 °C on 24-well plates (3×10^4 cells/well density) and grown to confluence, as previously described (Mossmann, 1993). Then, cells were treated with increasing pesticide concentrations: PQ (50, 100, 250, 500 and 1000 μM), MB (3, 6, 15, 30 and 60 μM) or with their combinations (PQ 50 μM + MB 3 μM; PQ 100 μM + MB 6 μM; PQ 250 μM + MB 15 μM; PQ 500 μM + MB 30 μM; PQ 1000 μM + MB 60 μM), for 24 or 48 h.

Thereafter, medium was removed and replaced with fresh medium containing MTT (0.5 mg/ml) and cells were allowed to incubate in the dark for 3 h at 37 °C. After supernatant removal, a dimethyl sulfoxide (DMSO)-ethanol (EtOH) (4:1) mixture was added to each well to dissolve formazan crystals. The optical densities (OD) were then read using a microplate spectrophotometer (GENios Tecan) at 590 nm and the results were expressed as a percentage of the OD value of treated cell cultures compared to untreated ones.

Based on MTT test results, the following concentrations were used to assess the effects of cell exposure to pesticides: 100 μM PQ, 6 μM MB or their combination for 48 h.

2.3. Western blot analysis

After 48 h exposure to pesticides, cells were washed with ice-cold phosphate buffered saline (PBS), and collected by centrifugation at 4 °C (2000× *g*, 5 min). Cells were lysed on ice with a buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA with freshly added protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and then stored at −80 °C. Protein concentrations were determined using BCA protein assay kit (Thermo Scientific, Milan, Italy). For each sample, 30 μg of proteins were separated on a 10% SDS-polyacrylamyde gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Milan, Italy). The membranes were incubated overnight at 4 °C in the blocking solution with the primary antibodies anti-TH polyclonal rabbit antibody (1:1500; Cell Signaling, Beverly, MA, USA) or anti-α-syn rabbit polyclonal antibody (1:500; GeneTex Inc., Texas, USA). Membranes were then washed three times and incubated for 1 h at room temperature with a horseradish peroxidase-linked anti-rabbit (1:3000) secondary antibody (Amersham Biosciences, Little Chalfont, UK). Equal loading was further ascertained by re-probing membranes with a mouse anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:2000; Millipore Corporation, Billerica, MA, USA) and with a horseradish peroxidase-linked anti-mouse secondary antibody (1:3000). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences Corp., Milan, Italy). The relative density of the immunoreactive bands was quantified by densitometry using ImageJ 1.383 software (NIH, MA, USA) on a Versadoc MP 4000 System (Bio-RAD Imaging Densitometer). Data are presented as mean ± SEM of the optical density ratios, normalized to GAPDH levels used as equal loading control, and expressed as percentage of control untreated cells.

2.4. Real-time quantitative RT-PCR

After 48 h exposure to PQ and MB or their combination, total RNA was isolated using TRIZOL reagent (Life Technologies, USA) according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The integrity of RNA was checked by 1% agarose gel electrophoresis and RNA concentrations were measured by spectrophotometry (only RNA samples with an OD260/OD280 ratio >2 were used). Total RNA was reverse transcribed with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) using random hexamers (0.75 μg of total RNA in a final reaction volume of 20 μl). Relative abundance of each mRNA species was assessed by real-time qRT-PCR using TaqMan Gene expression Master Mix for proteasome subunits gene expression analysis or using SYBR Green PCR Master Mix (Applied Biosystems, by Life Technologies, Foster City, CA, USA) for opioid receptor gene expression evaluations, in a Step One Real-Time PCR System (Applied Biosystems). All samples were run in triplicate. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2^{-DDCt}) for statistical analysis (Livak and Schmittgen, 2001). All data were normalized to the endogenous reference gene,