Comparative Biochemistry and Physiology, Part C 201 (2017) 51–57

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Permethrin pesticide induces NURR1 up-regulation in dopaminergic cell line: Is the pro-oxidant effect involved in toxicant-neuronal damage?

Laura Bordoni[a], Donatella Fedeli[b], Cinzia Nasuti[b], Melania Capitani[c], Dennis Fiorini[d], Rosita Gabbianelli[b,*]

[a] Schools of Advanced Studies, University of Camerino, 62032 Camerino, MC, Italy
[b] School of Pharmacy, University of Camerino, 62032 Camerino, MC, Italy
[c] Translational Gastroenterology Unit, University of Oxford, Oxford, UK
[d] School of Science and Technology, University of Camerino, 62032 Camerino, MC, Italy

ARTICLE INFO

Keywords:
PC12
Permethrin
Nurr1
Antioxidants
Redox state

ABSTRACT

The mechanisms associated to the development of neurodegeneration due to pesticide exposure are not clear yet. In this study we evaluated how permethrin pesticide (PERM) can influence the Nurr1 gene and protein expression, and if a pro-oxidant activity of the pesticide contributes to up-regulation of Nurr1 in a dopaminergic cell line. Incubation of PC12 cells with 1 μM PERM for 72 h, leads to over expression of *Nurr1* gene. This effect occurs with both corn oil and extra virgin olive oil (EVO) used to solubilize the toxicant. In order to investigate if the *Nurr1* up-regulation induced by PERM, was associated to the pro-oxidant activity of the pesticide, antioxidants as glutathione (GSH), tocotrienols (TOC) and Electrolyzed Reduced Water (ERW) were tested. RT-PCR of *Nurr1* showed that its up-regulation was significantly reduced in the presence of antioxidants, especially by addition of ERW. Western-blot analysis reveals that ERW was able to counterbalance the up-regulation of Nurr1 protein induced by permethrin exposure.

1. Introduction

Meta-analysis of idiopathic Parkinson's disease (PD) and pesticide exposure reveal a correlation between pesticide toxicity and the onset of dopaminergic neurodegeneration (Ahmed et al., 2017; Priyadarshi et al., 2000; Van Maele-Fabry et al., 2012). However, the mechanisms related to the neuronal damage are complex and not completely identified, suggesting that more factors are responsible for the development of neurodegeneration. Considering the high prevalence of idiopathic PD (representing the 85–90% of the disease) and that PD also occurs in subjects that have not a family history for the disease, it makes to think that the role of environmental factors in the modulation of genetic and epigenetic responses has to be considered (Ahmed et al., 2017).

Exposure to toxicant chemicals contained as residues in food as well as in drinking water and/or environmental metals like Mg, Cu and Fe, contribute to PD development: people living in rural area are more exposed to environmental stress and finally to neuronal damage (Allen and Levy, 2013; Lopert and Patel, 2016). Furthermore, post-mortem brain tissues from PD patients show inhibition of mitochondrial complex I activity, increased oxidative stress at the level of lipids, proteins and DNA and decrease in antioxidant capacity and glutathione (GSH) levels, considered typical biomarkers of disease (Allen and Levy, 2013; Lopert and Patel, 2016).

Studies on animal model have been carried out to investigate the mechanisms associated to pesticide-induced neurodegeneration (Fedeli et al., 2017; Nasuti et al., 2017; Vincenzetti et al., 2016). On this basis, early life exposure to permethrin pesticide (PERM) during brain development revealed that PERM at low dosage is able to induce a progressive Parkinson-like disease in animals (Fedeli et al., 2017; Nasuti et al., 2017; Vincenzetti et al., 2016). Changes in oxidative biomarkers at protein, lipid and DNA level have been observed together with an unbalance of endogenous antioxidant enzymes and low GSH content in treated animals (Gabbianelli et al., 2013; Carloni et al., 2013; Gabbianelli et al., 2009; Falcioni et al., 2010). After all, increased dopamine turnover leading to low level of dopamine in midbrain, contributes to reactive oxygen species (ROS) production (Nasuti et al., 2007; Nasuti et al., 2013; Carloni et al., 2012). Furthermore, neonatal toxicant exposure promotes also early up-regulation of Nurr1 together with compensative α-synuclein over expression. A direct effect of toxicant on *Nurr1* gene regulation could be hypothesized since PERM can cross the blood-brain barrier remaining longer in the brain, and molecular docking identified nine sites of interaction of PERM on Nurr1

* Corresponding author at: School of Pharmacy, University of Camerino, 62032 Camerino, MC, Italy.
E-mail address: rosita.gabbianelli@unicam.it (R. Gabbianelli).

http://dx.doi.org/10.1016/j.cbpc.2017.09.006
Received 25 July 2017; Received in revised form 15 September 2017; Accepted 20 September 2017
Available online 22 September 2017
1532-0456/

protein (Fedeli et al., 2017; Decressac et al., 2012). In addition, early life exposure to PERM has been associated to intergenerational effects: PERM-exposed parents generate a frequency of 40% of male and 50% of female offspring with the same *Nurr1* overexpression at adolescent age of their exposed parents as well as the same global DNA methylation identified in their early-life treated mothers (Bordoni et al., 2015).

In this complex scenario, our study aims to evaluate if the overexpression of *Nurr1*, a transcription factor involved in the regulation of the development and the survival of dopaminergic neurons, is associated with redox system alteration. Understanding the mechanism behind the regulation of *Nurr1* gene due to pesticide could open a way to test new compounds able to counterbalance the damage from early stage of the disease in the animal model of progressive PD (Fedeli et al., 2017; Nasuti et al., 2017; Vincenzetti et al., 2016; Gabbianelli et al., 2013; Carloni et al., 2013; Gabbianelli et al., 2009; Falcioni et al., 2010; Nasuti et al., 2007; Nasuti et al., 2013; Carloni et al., 2012; Fedeli et al., 2012). To reach this goal *Nurr1* gene expression was evaluated in vitro using a rat dopaminergic cell line, PC12 pheochromocytoma cells. These cells are able to express *Nurr1*, that is preferentially induced by depolarization, but not by nerve growth factor or epidermal growth factor (Liu et al., 2003). Considering that PERM neurotoxicity depends on its capacity to bind specially sodium channels leading to membrane depolarization (Soderlund, 2012), and that also hydroperoxides and increase of oxidized glutathione (GSSG) mediate membrane depolarization (Scott et al., 1987; Tretter and Adam-Vizi, 1996), PC12 cells were used to investigate the mechanisms associated to *Nurr1* overexpression, with a particular focus on the role of redox system alteration in this pathway. To this aim, PC12 cells were treated with PERM, in presence or not of compounds with antioxidant capacity as GSH, tocotrienols (TOC) and Electrolyzed Reduced Water (ERW). Nurr1 upregulation and free radical production due to PERM toxicity in dopaminergic cell line were analysed in order to investigate dopaminergic pathway and oxidative markers.

2. Materials and methods

Technical grade (75:25, *trans:cis* 92.4% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate, PERM, (NRDC 143) was generously donated by Dr. A.Stefanini of ACTIVIA, Milan, Italy. Corn oil, glutathione (GSH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxydopamine, deoxycolic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Sodium Dodecyl Sulphate (SDS), diphenyl-1-pyrenylphosphine (DPPP), NP-40 were purchased from Sigma-Aldrich. L-glutamine was obtained by PAA Laboratories GmbH/Austria. Dulbecco's Modified Essential Medium (DMEM), Fetal Bovine serum (FBS), Penicillin/Streptomycin (P/S) were purchased from Corning.

Extra Virgin Olive oil (EVO) from the “Raggia” variety, was produced in 2015 in Montegranaro, (Marche region, Central Italy), and the physicochemical characteristics were the following: acidity 0.21% oleic acid, peroxide index 6.2 meq/kg oil, oleic acid:linoleic acid ratio 14.6, α-tocopherol 284,3 mg/kg, polar polyphenols 632.7 mg/kg.

2.1. Cell culture and treatment

PC12 cells, *Rattus norvegicus* dopaminergic neuronal phenotype derived from an adrenal tumour, were used as a paradigm for neurobiological and neurochemical studies. These cells possess all enzymes involved in dopamine synthesis and they represent a simply model of neuronal degeneration treated with toxicants (Grau and Greene, 2012). PC12 cells were seeded at a density of 1.5×10^6 in 25 cm^3 flask, cultured in a humid atmosphere (5% CO2, 37 °C) in DMEM supplemented with 10% Fetal Bovine serum, (FBS), 1% Penicillin/Streptomycin (P/S) and 1% glutamine. PC12 cells were incubated 72 h with permethrin dissolved in corn oil or extra virgin olive oil (EVO) at the concentration of 1 μM in order to reach a percentage of oil in the cell medium of 0,1%, and treated with test substances (32 nM GSH and 1 μM tocotrienol extracted from *Elaesis guineensison* oil Vertuani et al., 2004) in sterile distilled water, ERW 100 μl/ml, and 70 μM 6-hydroxydopamine (6-OHDA) in the presence or absence of 1 μM permethrin. Various concentrations and time of incubation (6 h, 24 h,48 h and 72 h) were studied in the presence of various concentrations of permethrin (from 0.01 μM to 10 μM) to identify the lower amount of toxicant able to provoke an effect of Nurr1 gene express. 1 μM was chosen as the lower able to modulate Nurr1 gene expression.

2.2. Generation of electrolyzed reduced water (ERW)

ERW was produced by the continuously electrolyzing apparatus (Chanson revolution 9 plates, Taiwan), wherein tap water was the main water source. ERW was prepared by physical filtering followed by electrolysis and collected in a cell equipped with a cathode platinum-coated titanium electrode. ERW was adjusted to pH 7.4 and oxidative-reduction potential (ORP) of − 300 mV corresponding to 400 ppb molecular hydrogen.

2.3. RNA extraction and RT-PCR

After 72 h of incubation, PC12 cells were collected and total RNA was extracted using RNA Isolation kit (NucleoSpin RNA Purification Kit, Macherey Nagel) according to the manufacturer's instructions. RNA quality was checked by spectrophotometric analysis (OD260/280), while its quantity was measured using the OD260 by Nano-Drop spectrophotometer. Reverse Transcription reactions were performed using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio—Rad Inc., USA) according to the manufacturer's instructions. RT-PCR was employed to evaluate mRNA expression of genes of interest. The following specific primers were designed on the basis of gene and mRNA sequences available online (http://www.ncbi.nlm.nih.gov/gene) and purchased from Metabion (Metabion International AG, Germany): b-Actin, TAAAGACCTCTATGCCAACACAGTGC (fw) and AGAGTACTTG CGCTCAGGAGGAG (rv); Nurr-1, GGTTTCTTTAAGCGCACGGTG (fw) and TTCTTTAACCATCCCAACAGCCAG (rv). qPCR analysis was performed in a total volume of 20 μl containing 50 ng of template cDNA, 0.25 μM sense and antisense primers, 10 μl of iQ SYBR Green Supermix (Bio-Rad Inc., USA) by using a CFX96 Real-time PCR detection system (Biorad Inc.,USA).

2.4. Protein cell extraction for western blot analysis

After 72 h of incubation, cells were collected and lysed using RIPA/SDS buffer (1% NP40, 0.5% Na-deoxycolic acid and 0.1% SDS in phosphate buffer saline) with freshly added protease inhibitors (Fedeli et al., 2012). The lysates were passed several times through a 22-gauge needle and the releasing of the proteins, followed by the ice incubation for 30 min, and vortexed every 5 min. Samples were then centrifuged for 20 min at 4 °C at 14000 rpm, and the supernatant containing the proteins was collected. Proteins concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific).

2.5. Nurr1 and tyrosine hydroxylase expression by Western Blot analysis

20 μg for tyrosine hydroxylase or 40 μg for Nurr 1 protein samples were loaded on SDS–PAGE 10% along with molecular weight markers, with a range between 250 and 4 kDa (Thermo Scientific Spectra Multicolor Broad Range protein Ladder). After the gel electrophoresis, proteins were electro-blotted on nitrocellulose support (PVDF Transfer Membrane 0.45 μM, Thermo Scientific). Reactive sites were blocked by incubating the membrane for 1 h at room temperature with 5% non fat dry milk (Santa Cruz Biotechnology Inc., USA) dissolved in PBS. After two washing with PBS-Tween 0.05%, the membrane was incubated