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Alterations in mitochondrial dynamics induced by tebufenpyrad and pyridaben in a dopaminergic neuronal cell culture model

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ABSTRACT

Tebufenpyrad and pyridaben are two agro-chemically important acaricides that function like the known mitochondrial toxicant rotenone. Although these two compounds have been commonly used to kill populations of mites and ticks in commercial greenhouses, their neurotoxic profiles remain largely unknown. Therefore, we investigated the effects of these two pesticides on mitochondrial structure and function in an in vitro cell culture model using the Seahorse bioanalyzer and confocal fluorescence imaging. The effects were compared with rotenone. Exposing rat dopaminergic neuronal cells (N27 cells) to tebufenpyrad and pyridaben for 3 h induced dose-dependent cell death with an EC_{50} of 3.98 μM and 3.77 μM, respectively. Also, tebufenpyrad and pyridaben (3 μM) exposure induced reactive oxygen species (ROS) generation and m-aconitase damage, suggesting that the pesticide toxicity is associated with oxidative damage. Morphometric image analysis with the MitoTracker red fluorescent probe indicated that tebufenpyrad and pyridaben, as well as rotenone, caused abnormalities in mitochondrial morphology, including reduced mitochondrial length and circularity. Functional bioenergetic experiments using the Seahorse XF96 analyzer revealed that tebufenpyrad and pyridaben very rapidly suppressed the basal mitochondrial oxygen consumption rate similar to that of rotenone. Further analysis of bioenergetic curves also revealed dose-dependent decreases in ATP-linked respiration and respiratory capacity. The luminescence-based ATP measurement further confirmed that pesticide-induced mitochondrial inhibition of respiration is accompanied by the loss of cellular ATP. Collectively, our results suggest that exposure to the pesticides tebufenpyrad and pyridaben induces neurotoxicity by rapidly initiating mitochondrial dysfunction and oxidative damage in dopaminergic neuronal cells. Our findings also reveal that monitoring the kinetics of mitochondrial respiration with Seahorse could be used as an early neurotoxicological high-throughput index for assessing the risk that pesticides pose to the dopaminergic neuronal system.

1. Introduction

A growing body of evidence suggests that exposure to neurotoxic pesticides in agricultural settings is associated with increased risk for developing Parkinson's disease (PD) (Baltazar et al., 2014; Freire and Koifman, 2012; Parron et al., 2011). Among the pesticides associated with PD, rotenone is a well-characterized inhibitor of mitochondrial complex I that occurs naturally in tropical legumes (Cabeza-Arvelaiz and Schiestl, 2012; Greenamyre et al., 2001). Experimentally, exposure to rotenone was shown to reliably produce Parkinson's-like pathology in various animal models of PD (Betarbet et al., 2000; Greenamyre et al., 2010; Johnson and Bobrovskaya, 2015; Testa et al., 2005). Furthermore, recent epidemiological evidence has linked human rotenone exposure with PD (Spivey, 2011; Tanner et al., 2011).

The pathogenic mechanisms underlying rotenone-induced Parkinsonism are not fully understood, but possibly involve inhibition of mitochondrial respiratory chains and induction of oxidative damage (Johnson and Bobrovskaya, 2015; Sherer et al., 2003). Mitochondria are pivotal to the homeostatic functioning of cells and thus the central nervous system. The primary role of mitochondria is to provide energy to cells via oxidative phosphorylation (Chan, 2006; Hoppins et al., 2007; Jin et al., 2014a; Zhang and Chan, 2007). Some of the critical biochemical

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abnormalities resulting from mitochondrial dysfunction are increased generation of reactive oxygen species (ROS), loss of ATP production during cellular respiration and impaired Ca^{2+} ion channels (Schapira, 2007; Winklhofer and Haass, 2010). Neurotoxic stress also induces structural damage to mitochondria including mitochondrial fragmentation and mitophagy (Lin et al., 2012; Lin and Beal, 2006).

Tebufenpyrad (IUPAC name: N-[(4-tert-butylphenyl)methyl]-4-chloro-5-ethyl-2-methylpyrazole-3-carboxamide) and pyridaben (IUPAC name: 2-tert-butyl-5-[(4-tert-butylphenyl)methylsulfanyl]-4-chloropyridazin-3-one) are common acaricides used to kill populations of mites and ticks in commercial greenhouses. Tebufenpyrad is chemically classified as a pyrazole carboxyamide, which is registered for use in greenhouses for the protection of ornamental plants (EPA PC Code – 090102). Pyridaben is chemically classified as a pyridazinone, whose major application is in greenhouses and vineyards (EPA PC Code – 129105). Similar to rotenone, tebufenpyrad and pyridaben have been shown to function as mitochondrial complex I inhibitors (classified by the IRAC-Insecticide Resistance Action Committee – http://www.irac-online.org/modes-of-action/). Although their intended mode of action and target toxicity are similar to those of rotenone, both tebufenpyrad and pyridaben have not been studied in detail with respect to their neurotoxicity. Therefore, in this study, we evaluated the neurotoxic effects of tebufenpyrad and pyridaben in rat dopaminergic neuronal cells, with particular emphasis on their effects on mitochondrial dynamics and their roles in dopaminergic neuronal cell death.

2. Materials and methods

2.1. Chemicals

We purchased tebufenpyrad (96% purity) from AK Scientific Inc. (Union City, CA), pyridaben (99.1% purity) from Chem Services (West Chester, PA), and rotenone (95–98% purity) and hydrogen peroxide (30 wt. % in H_2O) from Sigma (St. Louis, MO). DMSO was purchased from Fisher Scientific (Fair Law, NJ). We purchased RPMI 1640 media, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and Sytox Green nucleic acid fluorescence stain from Molecular Probes (Eugene, OR), the Muse® Count &Viability assay kit (Catalog # MCH100102) from EMD Millipore (Billerica, MA), and the 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) fluorescent probe and MitoTracker red CMXROS and MitoTracker Green dyes from Invitrogen (Carlsbad, CA). The Cell Titer 96® AQueous Non-Radioactive Cell Proliferation assay kit and Cell Titer Glo Luminescent Cell Viability assay kit were bought from Promega (Madison, WI). The Aconitase assay kit was purchased from Abcam (Cambridge, MA). Oligomycin, hydrogen peroxide, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) and antimycin A were purchased from Sigma, and the Seahorse FluxPak calibration solution was bought from Seahorse Biosciences (Billerica, MA).

2.2. Cell culture and treatment paradigm

The rat immortalized mesencephalic dopaminergic neuronal cell line ($1RB_3AN_{27}$, also known as N27 cells) was a kind gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). These N27 cells have the potential to differentiate and produce dopamine in culture when exposed to a suitable cAMP triggering agent, and once the cells are differentiated they possess increased tyrosine hydroxylase (TH) expression and dopamine levels (Adams et al., 1996; Zhang et al., 2007). In this study, undifferentiated cells were grown in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 50 units of penicillin, and 50 μg/ml streptomycin, as described previously (Anantharam et al., 2002; Jin et al., 2011b; Prasad et al., 1998). In general, cells were plated in a tissue culture plate or flask in accordance to the experimental requirements and were cultured overnight in a humidified atmosphere of 5% CO_2 at 37 °C. The cell density plated for each experiment has been provided in the methods section. The cells were treated with the specified concentrations of tebufenpyrad and pyridaben for 0–3 h in serum-free RPMI media. For all experiments with N27 cells, treatments were performed when the cells were 65–70% confluent. The pesticides tebufenpyrad and pyridaben are lipophilic in nature and are hence dissolved in DMSO. In the ROS generation and aconitase activity experiments, cells were treated with 100 μM H_2O_2 for 45 min as a positive control. Similarly, 1 μM rotenone for 3 h was used as a positive control in the SYTOX Green assay, Muse Annexin V/7-AAD assay, ATP production, and the mitochondrial dysfunction and damage studies.

2.3. MTS cell viability assays

Cell viability was measured using the Cell Titer 96® AQueous Non-Radioactive Cell Proliferation (MTS assay) kit from Promega as described previously (Jin et al., 2014b). Briefly, N27 cells were plated at 0.8×10^4 cells/well in 96-well plates one day before treatment. The next day cells were treated in serum-free RPMI media with different concentrations of tebufenpyrad or pyridaben (0, 1, 2, 3, 5, 7, 9, 10 and 30 μM) for a time period of 3 h. Following treatment, 10 μl of MTS solution reagent mix was added to each plate well and incubated at 37 °C for 45 min. At the end of incubation, the formazan crystals that formed in the live cells were dissolved by adding 25 μl of DMSO to each well. Finally, readings were taken at a wavelength of 490 nm and another reference reading for each well was taken at 670 nm to eliminate background. The data were then plotted as a dose–response curve (depicting the EC_{50}) using Prism 4.0 (GraphPad Software, San Diego, CA).

2.4. SYTOX Green cytotoxicity assays

Cell death after exposing the N27 cells to 3 μM of tebufenpyrad or pyridaben was determined using the SYTOX Green cytotoxicity assay, as previously described (Jin et al., 2011a; Latchoumycandane et al., 2011). The SYTOX Green dye only permits dead cells to produce green fluorescence. In brief, N27 cells were grown in 24-well cell culture plates (4×10^4 cells/well) and treated with 3 μM of tebufenpyrad or pyridaben along with 1 μM SYTOX Green dye for 3 h. Fluorescent images were then taken using fluorescence microscopy (Cytation 3, Biotek, Winooski, VT) that was coupled with a 40× objective and Gen5 imaging software. For further validation, the green fluorescence was quantitatively measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm with the use of a fluorescence microplate reader (Cytation3, Biotek).

2.5. Annexin V/7-AAD apoptosis assay

The cytotoxic effects of tebufenpyrad and pyridaben on N27 cells were also analyzed with the Annexin V/7-AAD assays using the Muse® Annexin V and Dead Cell assay kit from Millipore (Billerica, MA). Annexin V, which is membrane permeable, labels all cells containing a nucleus. The second component of the kit is 7-AAD, which stains the membrane of the cells that have been compromised and are dying or dead. This synergistic functioning of the two components differentiates between cell populations based on their health (Khan et al., 2012; Marusiak et al., 2014). The assay was performed according to manufacturer's protocol. Briefly, N27 cells were grown in 6-well culture plates (3×10^5 cells/well) and

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