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Fluazinam targets mitochondrial complex I to induce reactive oxygen species-dependent cytotoxicity in SH-SY5Y cells

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ABSTRACT

Although the underlying cause of Parkinson's disease (PD) is not well characterized, epidemiological studies suggest that exposure to agricultural chemicals is a risk factor for PD. Fluazinam (FZN) is a new active ingredient for the control of grey mould, belonging to the novel broad spectrum phenylpyridinamine fungicides. We used human neuroblastoma SH-SY5Y cells to investigate mechanisms of dopaminergic cell death in response to FZN. FZN treatment produced dose-dependent cytotoxicity, and decreased the tyrosine hydroxylase (TH) expression in SH-SY5Y cells. We provided evidence for the occurrence of oxidative stress and oxidative damage during FZN exposure on dopaminergic cells through the measurement of reactive oxygen species (ROS) in cells with DCFH-DA. The cytotoxic effects of FZN appear to involve an increase in ROS generation since pretreatment with *N*-acetyl cysteine (NAC), an anti-oxidant, reduced cell death. After FZN treatment, dopamine (DA) levels decreased in both cell and culture media, and oxidative effects of FZN were blocked by NAC pretreatment. We show that cell death in response to FZN was due to apoptosis since FZN exposure results in an increased in cytochrome c release into the cytosol and activated caspase-3 through p38 and JNK signaling. Furthermore, the blocking of p38 or JNK signaling inhibits FZN-induced cell death. Phosphorylation of mitogen-activated protein kinases precedes cytochrome c release and caspase-3 activation. This cellular response is characteristic of mitochondrial dysfunction. Therefore, we also investigated the effect of FZN on mitochondrial complex I activity in FZN-treated cell. Interestingly, we show that FZN inhibited the complex I activity. Thus in this study, we report a new mode of action by which the fungicide FZN could triggers apoptosis.

1. Introduction

Accumulating evidence suggests that environmental toxins are major contributing factors to the development of Parkinson's disease (PD), coupled with genetic predispositions (Ramsden et al., 2001). Exposure to agricultural chemicals in a rural environment, drinking well-water, and occupational exposure have been postulated to be environmental risk factors for the disease (Hatcher et al., 2008). Exposure to pesticides such as rotenone and paraquat (PQ) plays an important role in the pathogenesis of PD via reactive oxygen species (ROS) generation and mitochondrial complex I dysfunction (Choi et al., 2008; Kang et al., 2009) and enhanced oxidative stress are important role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and PD (Siminian and Coyle, 1996). There is substantial evidence indicating elevation of oxidative stress during pesticide poisoning including increased lipid peroxidation, diminished energy metabolism and decreased cytochrome oxidase activity (DiCiero Miranda et al., 2000). Dopaminergic neurons may be preferentially targeted by pesticides such as PQ because of their vulnerability to reactive oxygen species-mediated oxidative injury (Bonneh-Barkay et al., 2005). Compared to other neuronal cells, dopaminergic cells are much more sensitive to oxidative injury (Dinis-Oliveira et al., 2006; Lotharius and O'Malley, 2000).

Mitochondria are considered to be the main link between cellular stress signals activated during short- and long-term neuronal cell injury, leading to apoptotic cell death (Jordán et al., 2003; Marttson and Kroemer, 2003). Apoptotic events can cause an increase in the permeability of the outer mitochondrial membrane (Green, 2006). This process triggers the release of intermembrane space proteins into the cytoplasm, including cytochrome c, Smac/DIABLO, and apoptosis-inducing factor (Green and Kroemer, 2004).

A variety of extracellular stimuli elicit cellular activities such as survival, proliferation, differentiation, and apoptosis through the activation of a family of mitogen-activated protein kinases (MAPKs) consisting of the extracellular signal-regulated kinases (ERKs), the p38 MAPK, and the c-Jun N-terminal kinases (JNKs) (Davis, 2000; Johnson and Lapadat, 2002). JNK is activated in response to many different stress factors including heat shock, inflammatory cytokines, protein synthesis inhibitors, growth factor withdrawal, chemotherapeutic

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drugs, and ultraviolet irradiation (Kyriakis and Avruch, 2001; Gallo and Johnson, 2002). JNK activation has been implicated in both cell survival and cell death in response to stress depending on the cell type or the stimulus (Davis, 2000; Kyriakis and Avruch, 2001). Several studies have suggested that the JNK and p38 signal transduction pathway may be involved in neurodegeneration and that blocking the activity of the JNK and p38 MAP kinases may be effective in preventing disease-related neuropathology (Peng and Andersen, 2002; Caughlan et al., 2004; Newhouse et al., 2004).

Fluazinam (FZN) is a preventative fungicide from the pyridinamine group that was introduced in the 90s and quickly established itself as a new standard for the control of blight caused by phytophthora infestans in potatoes (Anema and Bouwman, 1992). However, while FZN has been reported to cause a toxic response in some organs (van Ginkel and Sabapathy, 1995; Draper et al., 2003), the molecular mechanisms underlying FZN-induced neurotoxicity are not well understood.

Inhibition of mitochondrial complex I activity may contribute to the neurodegenerative process in PD. Dopaminergic neurotoxins enhance production of ROS in mitochondria. In this study, we sought to define the mode of cell death induced by FZN in a human dopaminerigc cell line SH-SY5Y and to elucidate the underlying signal transduction pathways mediating FZN-induced cell death.

2. Materials and methods

2.1. Reagents and antibodies

FZN (Sigma–Aldrich, MO) was dissolved in DMSO. TH, JNK, p-JNK, p38, p-p38 and caspase-3 were purchased from Cell Signaling Technologies, β-actin from Abcam and cytochrome c from Biovision. All other chemicals were obtained from Sigma–Aldrich.

2.2. Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells used for Western blot analysis were grown in 6-well cluster dishes, whereas those used for cell viability assays were grown in 96-well plates. Cells were plated at a density of 5×10^4 cells (96 well plate) and cultured for 24 h. Cells were serum-starved for 24 h prior. FZN stock (1 mM) used to make the dilutions for cell treatment. Immediately before treatment FZN addition, dilutions of FZN were made in DMSO and added to fresh cell medium to achieve the required concentration.

2.3. Cell viability

Cell viability was measured by MTS assay (CellTiter96® AQueous One Solution Cell Proliferation Assay, Promega, WI). Briefly, MTS was added to SH-SY5Y cells in 96-well plates and the plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. Metabolically active cells convert the yellow MTS tetrazolium compound to a purple formazan product. The latter is soluble in tissue culture medium and the quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. Results are expressed as percentages of controls.

2.4. Measurement of intracellular reactive oxygen species

Production of ROS was measured using an oxidation-sensitive fluorescent probe, 2,7-dichlorofluorescin diacetate (DCFH-DA), based on the ROS-dependent oxidation of DCFH-DA to DCF. Cells plated in coated 6-well plates were grown in DMEM medium and treated with 100 μM FZN or DMSO as control for 6 h, with or without pre-treatment with the antioxidant, *N*-acetyl cysteine (NAC). The medium was removed and cells were washed with PBS. Then, 200 μl DCF H-DA (10 μM) was added for 30 min at 37 °C in the dark and the cells were washed with PBS. Intracellular ROS production was measured based on the fluorescence intensity. Fluorescent images were taken with an Olympus microscope.

2.5. DAPI staining

Cells were first cultured on 35 mm glass bottom culture dishes. After treatment with FZN, cells were fixed by incubation in 4% PFA for 30 min. Following washing in PBS, the cells were incubated in 1 g/mL DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope.

2.6. Western blot analysis

To determine the levels of protein expression, we prepared extracts from the SH-SY5Y cells. Adherent cells were scraped off the culture dishes and lysed by incubation with the radio-immunoprecipitation assay lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and phosphatase inhibitor cocktail (Roche, IN, USA) on ice. Collected cells were broken by sonication on ice and centrifuged at 10,000*g* for 20 min at 4 °C. Protein concentrations were determined with Bradford reagent and 30 μg samples of extracted protein were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated in the presence of different primary antibodies at 4 °C overnight and then the membranes were incubated with secondary antibody coupled to horseradish peroxidase. Immunoreactivity was visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, England, UK). Protein bands were quantified with a densitometer (Molecular Devices, VERSAmax, CA).

2.7. Cell fractionation

Cells were lysed in buffer A (0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF) by pestle homogenizer on ice. Homogenates were centrifuged at 750*g* for 10 min at 4 °C and supernatants were collected and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatants were used as the cytosolic fraction, and the pellet was used as the mitochondrial fraction. The pellets were resuspended in buffer B (0.25 M sucrose, 10 mM Tris–HCl (pH7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 1% NP40). To prepare nuclear extracts, the cells were washed twice with cold PBS and detached from plates with detaching buffer (150 mM NaCl, 1 mM EDTA (pH8.0), 40 mM Tris–HCl (pH7.6)) for 5 min at room temperature. The cells were then transferred to microcentrifuge tubes and centrifuged at 300*g* for 4 min at 4 °C. The supernatants were discarded, and the pellets were resuspended in 400 μl cold buffer A (10 mM Hepes (pH7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 15 min. 10 μl of 10% Nonidet P-40 was added, and the mixtures were vortex briefly. Nuclei were pelleted by centrifugation at 2800*g* for 4 min at 4 °C and then resuspended in 50 μl of ice-cold buffer B (20 mM Hepes (pH7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). The