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Fluazinam-induced apoptosis of SH-SY5Y cells is mediated by p53 and Bcl-2 family proteins

Jeong Eun Lee [a], Jin Sun Kang [a], In Chul Shin [a], Soo-Jin Lee [b], Dong-Hoon Hyun [c], Kyung Suk Lee [d], Hyun Chul Koh [a,*]

[a] Department of Pharmacology, College of Medicine, Hanyang University, Republic of Korea
[b] Department of Occupational & Environmental Medicine, College of Medicine, Hanyang University, Republic of Korea
[c] Department of Life Science, Ewha Womans University, Republic of Korea
[d] Rural Development Administration, National Academy of Agricultural Science, Republic of Korea

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ABSTRACT

A number of epidemiological studies have demonstrated a strong association between the incidence of neurodegenerative disease and pesticide exposure. Fluazinam (FZN) is a preventative fungicide from the pyridinamine group that was introduced in the 1990s and that quickly established itself as a new standard for the control of blight caused by *Phytophthora infestans* in potatoes. We used human neuroblastoma SH-SY5Y cells to investigate mechanisms of neuronal cell death in response to FZN and showed that FZN was cytotoxic to SH-SY5Y cells in a concentration- and time-dependent manner. Additionally, we showed that FZN treatment significantly decreased the neuron numbers including dopaminergic neurons and mitochondrial complex I activity. The cytotoxic effects of FZN were associated with an increase in reactive oxygen species (ROS) generation because pretreatment with *N*-acetyl cysteine, an anti-oxidant, reduced cell death. We showed that neuronal cell death in response to FZN was due to apoptosis because FZN increased cytochrome C release into the cytosol and activated caspase-3 through the accumulation of p53. FZN also reduced the levels of Bcl-2 protein but increased the levels of Bax. Our results provide insight into the molecular mechanisms of FZN-induced apoptosis in neuronal cells.

1. Introduction

Accumulating evidence suggests that environmental toxins coupled with genetic predispositions are major contributory factors to the development of neurodegenerative disease (Ramsden et al., 2001). Exposure to agricultural chemicals in a rural environment, ingestion of well-water, and occupational exposure have been postulated to be environmental risk factors for environmental toxin-induced neurodegenerative disease (Hatcher et al., 2008). Furthermore, increasing evidence suggests an important role of exposure to pesticides such as rotenone and paraquat (PQ) in the pathogenesis of Parkinson's disease (PD) (Choi et al., 2008; Kang et al., 2009).

Reactive oxygen species (ROS) and other free radicals have been implicated in the pathogenesis of neurodegenerative diseases such as PD (Rao and Balachandran, 2002). The substantia nigra pars compacta (SNpc) region of the brain is able to oxidative stress because of its local environment. Furthermore, dopaminergic neurons may be preferentially targeted by pesticides such as PQ because of their vulnerability to ROS-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 the main link between cellular stress signals activated during short- and long-term neuronal cell injury and apoptotic cell death (Jordán et al., 2003; Mattson and Kroemer, 2003). Apoptotic events can cause an increase in the permeability of the outer mitochondrial membrane (Green, 2005). 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).

The pro-apoptotic Bcl-2 family proteins Bax and Bak have been shown to mediate this increase in permeability in many cell types. While the role of Bak in neuronal apoptosis is a matter of debate (Fannjiang et al., 2003), there is a large body of evidence showing that Bax is critically involved in neuronal cell death (Mladenović et al., 2004). The p53 protein has also been identified as a critical mediator of neuronal apoptosis (Duan et al., 2002; Jordán et al., 2003). p53-induced apoptosis proceeds through transcriptional induction of the BH3-only proteins PUMA and Noxa, Bax activation,

* Corresponding author. Tel.: +82 2 2220 0653; fax: +82 2 2292 6686.
E-mail address: hckoh@hanyang.ac.kr (H.C. Koh).

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and the mitochondrial release of cytochrome C (Cregan et al., 2004; Wyttenbach and Tolkovsky, 2006).

Fluazinam (FZN) was a preventative fungicide from the pyridinamine group that was introduced in the 1990s and that was quickly established itself as a new standard for the control of blight caused by *Phytophthora infestans* in potatoes. In subchronic and chronic toxicity studies, FZN was found to target the following organs: liver, lung, uterus, testes, pancreas, thymus, thyroid, stomach, eyes and brain (Health Canada, 2003). FZN caused dermatitis and asthma in human (van Ginkel and Sabapathy, 1995; Draper et al., 2003) and caused toxicity of liver and thyroid (Health Canada, 2003).

However, despite reports that FZN has toxic effects in some organs, the molecular mechanisms underlying FZN-induced neurodegeneration are not well understood.

To elucidate the molecular mechanisms underlying FZN cytotoxicity, we performed a detailed investigation of the link among FZN, oxidative stress, and the inductions of p53 and Bcl-2 family proteins in human neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, VA) and were 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. We performed the experiments using cells that had undergone fewer than 15 passages, and all studies were repeated several times with different batches of cells. Cells used for Western blot analysis were grown in six-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 were cultured for 24 h prior to treatment. To avoid inhibition of FZN, SH-SY5Y cells were starved for 24 h. FZN is a lipophilic compound, we were concerned that binding of FZN to serum proteins may compromise its activity. Cells were serum-starved for 24 h prior to treatment with FZN. Serum-starvation and DMSO did not affect the viability values of control plates.

2.2. Reagents and antibodies

FZN (Sigma–Aldrich, MO) was dissolved in DMSO. Bcl-2, Bax, p53, COX IV and caspase-3 antibodies were purchased from Cell Signaling Technologies, the β-actin antibody was obtained from Abcam (Cambridge, UK) and the cytochrome C antibody was purchased from Biovision (CA). Specific inhibitors of p53 and Bcl-2 were obtained from TOCRIS bioscience (Bristol, UK). All other chemicals were obtained from Sigma–Aldrich.

2.3. Drug treatment

FZN was obtained from Sigma–Aldrich, MO. 1 mM FZN stock used to make the dilutions for cell treatment. Immediately before treatment FZN addition, dilutions of FZN were made in dimethyl sulfoxide (DMSO) and added to fresh cell medium to achieve the required concentration.

Pifithrin-α and HA14-1 were dissolved in DMSO and were administered 30 min before FZN treatment. Serum starvation and addition of DMSO (0.005% final concentration) did not affect the viability values of control plates.

2.4. 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 the plate 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 were expressed as a percentage of the controls.

2.5. Measurement of LDH release

To measure the leakage of soluble cytoplasmic LDH into the extracellular medium due to cell death, a LDH cytotoxicity detection kit (Takara, MK401, Japan) was used. LDH converts pyruvate to lactic acid in the presence of reduced β-nicotinamide adenine dinucleotide (NADH), and any pyruvate not converted to lactic acid produces a brightly colored phenylhydrazone when treated with 2,4-dinitrophenylhydrazine. After incubation in the presence of either FZN or vehicle, culture medium was collected and centrifuged at $4000 \times g$ for 10 min at 4 °C. The resulting supernatant was used to measure LDH activity, following the manufacturer's instructions. The reaction was run in the dark for 30 min prior to measurement, and the absorbance was measured with a multiplate reader at 492 nm. Results are expressed as percentages of the control.

2.6. Immunocytochemistry

For immunostaining, cells were grown in 35 mm glass bottom culture dishes, fixed in 4% paraformaldehyde and incubated in blocking solution (10% bovine serum albumin and 0.3% triton X-100 with PBS) for 1 h at room temperature. Cells were stained with the monoclonal anti-MAP-2 antibody (Chemicon, Temecula, CA; 1:1000) prior to incubation with a biotinylated secondary anti-mouse. After 3 washes in PBS, the cells were incubated in appropriate Allexa 488 (1:400, Invitrogen, Carlsbad, CA) for 2 h at room temperature and mounted with Vectashield (Vector Labs, Burlingame, CA). Images of stained neurons were created using an Olympus microscope system.

2.7. Measurement of intracellular reactive oxygen species

Production of ROS was measured using an oxidation-sensitive fluorescent probe, 2,7-dichlorofluorescin diacetate (DCF-DA), based on the ROS-dependent oxidation of DCF-DA to DCF. Cells plated in coated six-well plates were grown in DMEM medium and treated with 5 μM FZN or DMSO as a control for 6 h, with or without pre-treatment with the antioxidant *N*-acetyl cysteine. The medium was removed and cells were washed with PBS. Then, 200 μl DCF-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 collected with an Olympus microscope.

2.8. 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, and 0.1 mM PMSF). Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C, and supernatants were collected and centrifuged at $10{,}000 \times g$ for 20 min at 4 °C. The supernatants were used as the cytosolic fraction, and the pellets were the mitochondrial fraction. The pellets were resuspended in buffer B (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, and 1% NP40).