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Dynamin-related protein 1 mediates mitochondria-dependent apoptosis in chlorpyrifos-treated SH-SY5Y cells

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ABSTRACT

Recent studies have demonstrated that dynamin-related protein 1 (Drp1), a mitochondrial fission protein, mediates mitochondria-dependent apoptosis through mitochondrial division. However, little is known about the mechanism by which Drp1 modulates apoptosis in response to chlorpyrifos (CPF)-induced toxicity. In this study, we determined that CPF-induced mitochondrial apoptosis is mediated by Drp1 translocation in SH-SY5Y human neuroblastoma cells. Our results showed that CPF treatment induced intrinsic apoptosis by activating caspase-9, caspase-3, and cytochrome *c* release in SH-SY5Y cells. Cytosolic Drp1 translocated to the mitochondria in CPF-treated cells and was phosphorylated at Ser616. Treating cells with CPF induced the generation of reactive oxygen species (ROS) and activation of mitogen-activated protein kinases (MAPKs). Inhibiting this ROS generation and MAPK activation abolished CPF-induced expression of phospho-Drp1. Furthermore, Drp1 was required for p53 to translocate to the mitochondria under CPF-induced oxidative stress. Treating cells with mitochondrial-division inhibitor-1 (mdivi-1), which blocks Drp1 translocation, increased the viability of CPF-treated cells by abrogating Drp1 translocation and caspase-3 activation. Specifically, pretreating cells with mdivi-1 inhibited Bax translocation to the mitochondria by blocking p53 signaling. Taken together, these data reveal a novel mechanism by which Drp1 activates mitochondrial-dependent apoptosis and indicate that inhibiting Dpr1 function can protect against CPF-induced cytotoxicity. We propose that inhibiting Drp1 is a possible therapeutic approach for pesticide-induced toxicity when hyperactivated Drp1 contributes to pathology.

1. Introduction

Mitochondria are critical subcellular organelles that govern energy generation, lipid metabolism, and Ca^{2+} buffering. A decline in mitochondrial activity has been implicated in a number of cellular processes, and dysfunctional mitochondria have been linked to the pathogenesis of a wide range of neurodegenerative disease, such as Parkinson's disease (PD) (Beal, 2005). Mitochondrial fission has significant implications in stress responses and apoptosis (Chan, 2012). Mitochondrial fission is controlled by mitochondrial fission proteins, such as dynamin-related protein 1 (Drp1), a member of the dynamin family of large GTPases. Drp1 is predominantly a cytosolic protein that forms oligomers, which are recruited to the outer mitochondrial membrane (OMM) and mediate the scission of mitochondrial membranes through GTP hydrolysis (Fukushima et al., 2001; Zhu et al., 2004). Drp1 activity is regulated by several post-translational modifications including phosphorylation (Elgass et al., 2013). Specifically, when phosphorylated at Serine 616, Drp1 is rapidly activated and stimulates mitochondrial fission during mitosis (Cho et al., 2013; Sanchis-Gomar and Derbré, 2014). Under stress conditions, Drp1 translocation to the mitochondria initiates mitochondrial fission and induces mitochondrial dysfunction, which subsequently leads to dissipation of mitochondrial membrane potential (MMP). As a consequence, the pro-apoptotic protein Bax translocates from the cytosol to mitochondrial membranes. This pathway is an early apoptotic event that correlates with mitochondrial fragmentation (Frank et al., 2001; Germain et al., 2005; Karbowski et al., 2002),

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release of the mitochondrial intermembrane space (IMS) protein cytochrome *c* to the cytosol, and increased apoptosis (Oettinghaus et al., 2012).

The tumor suppressor protein p53 is a transcriptional factor that mediates cell death. It initiates apoptosis through an intrinsic pathway by transcriptionally regulating the gene products of pro-apoptotic proteins (Agarwal et al., 1998). Under conditions of cell stress, a cytoplasmic pool of p53 mainly translocates to the OMM surface, where it behaves like a BH3-only protein, physically interacting with anti-apoptotic (Bcl-xL, Bcl-2, Mcl-1) and pro-apoptotic (PUMA, Bax, Bak) members of the Bcl-2 family. Bax expression is upregulated by p53, and Bax has been shown to be involved in p53-mediated apoptosis (Marchenko et al., 2000). Furthermore, recent studies suggest that p53 directly regulates mitochondrial fission through the activity of fission proteins such as Drp1. p53 can induce mitochondrial fission in HeLa cells by promoting Drp1 translocation to mitochondria through transcriptional suppression of miR-499. Previous studies have shown that p53-Drp1 binding caused increased mitochondrial fragmentation and mediated cell death in cardiomyocytes (Guo et al., 2013; Li et al., 2010; Wang et al., 2011a). However, the correlations between Drp1, p53, and Bax in chlorpyrifos (CPF)-treated cells are not well understood.

CPF, an acetylcholinesterase (AChE) inhibitor, is a widely used organophosphate pesticide. CPF is also used as a model pesticide to investigate the role of pesticide exposure in neuronal cell death (Caughlan et al., 2004). The neurotoxicity of CPF is caused by acetylcholinesterase inhibition in the central nervous system (CNS) (Chanda and Pope, 1996; Moser, 2000; Whitney et al., 1995). CPF is lipophilic molecule and therefore easily passes through the cell membrane into the cytoplasm. Recent studies have demonstrated that CPF generates oxidative stress and lipid peroxidation in different cell types and in a rat model and also causes neuronal damage by elevating the production of reactive oxygen species (ROS), DNA damage, and lipid peroxidation in the CNS (Geter et al., 2008; Saulsbury et al., 2009; Verma et al., 2007). We previously showed that CPF-induced apoptosis depends on ROS and signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK (p38), and extracellular signal-regulated kinase (ERK) 1/2, all of which have been implicated in apoptosis (Lee et al., 2012).

Based on these lines of evidence, we investigated the mechanisms of Drp1-dependent apoptosis in CPF-treated SH-SY5Y cells. We suggest that Drp1 is required for p53 translocation to the mitochondria under oxidative stress. We also found that blocking Drp1 function in cultured cells prevented CPF-induced, mitochondrial-dependent apoptosis *via* an interaction with p53 signaling. Furthermore, we suggest that the neuroprotective effects of mitochondrial-division inhibitor-1 (mdivi-1) are related to its ability to inhibit Drp1 activity. These data suggest that modulation of Drp1 activity is a possible therapeutic approach for pesticide-induced neurotoxicity when hyperactive Drp1 contributes to neurodegeneration.

2. Materials and methods

2.1. Cell culture

We obtained SH-SY5Y cells from the American Type Culture Collection (ATCC, VA) and cultured them in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. Cells were incubated at 37 °C under a saturated humidified atmosphere of 5% CO_2. Differentiation was induced by 10 μM Retinoic acid (RA) on day 1 after plating and continued for 7 days in accordance with previous study protocol (Lopes et al., 2010). All experiments were carried out 24 h after cells were seeded. Cells used for Western blot analysis were grown in 100π cluster dishes, and cells used for cell viability assays were grown in 96-well plates. Cells were plated at a density of 5×10^4 cells (96-well plates) and cultured for 24 h.

2.2. Reagents and antibodies

CPF (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO), which served as the vehicle control. A 40-mM CPF stock was diluted for cell treatments. Because CPF is lipophilic, we were concerned that CPF binding to serum proteins might compromise its activity; thus, cells were transferred to low-serum medium containing 0.1% FBS before CPF treatment in order to prevent excessive serum retention of CPF. To avoid possible inhibition of CPF by serum proteins, SH-SY5Y cells were starved for 24 h. Mdivi-1, Atropine, Mecamylamine, RA (Sigma-Aldrich, MO), SB 203580, SP 600125 (TOCRIS Bioscience, Bristol, UK), and U0126 (Cell Signaling Technologies, MA, USA) were dissolved in DMSO. SB 203580, SP 600125, and U0126 were administered 30 min before CPF treatment. Serum starvation and addition of DMSO (0.005% final concentration) did not affect the viability of control cells. Primary antibodies against JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, p38 MAPK, phospho-p38 MAPK, phospho-Drp1, caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, p53, Bax, COX IV, β-actin, and α-tubulin were purchased from Cell Signaling Technology. Tyrosine hyroxylase (TH) was purchased from Sigma-Aldrich. Glutamic acid decarboxylase 65 (GAD65) was purchased from Abcam (Cambridge, MA, USA). Drp1 antibodies were obtained from Bethyl Laboratories, Inc (Montgomery, TX), and cytochrome *c* antibodies were obtained from Bio Vision Technology (CA, USA).

2.3. Cell viability

Cell viability was measured with an MTS assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega, WI), based on the colorimetric conversion of yellow MTS tetrazolium to purple formazan. SH-SY5Y cells were plated in 96-well plates and incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were then treated with CPF for 24 h and incubated with MTS solution for 4 h. The MTS solution is soluble in tissue culture medium. The concentration of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells in culture. Data are expressed as percentage of the control.

2.4. Measurement of cytotoxicity

We used a lactate dehydrogenase (LDH) cytotoxicity detection kit to measure the leakage of soluble cytoplasmic LDH into the extracellular medium due to cell death (Takara, MK401, Japan). LDH converts pyruvate to lactic acid in the presence of reduced β-nicotinamide adenine dinucleotide (NADH). Pyruvate that is not converted to lactic acid produces a brightly colored phenylhydrazone when treated with 2, 4-dinitrophenylhydrazine. SH-SY5Y cells were plated in 24-well plates one day before the experiments. After incubation in either CPF or vehicle for 24 h, culture medium was collected and centrifuged at 4000 × *g* for 10 min at 4 °C. The LDH activity in the culture supernatant was measured after transferring the supernatant to 96-well plates. The reaction was run in the dark for 30 min before measurement, and the absorbance was measured at 490 nm with a multiplate reader. Results are expressed as a percentage of the control.

2.5. Western blotting analysis

To prepare whole protein lysates, cells were harvested with a cell lifter, and total proteins were isolated by incubating the cells