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Acrolein-mediated neuronal cell death and alpha-synuclein aggregation: Implications for Parkinson's disease

Abeje Ambaw[a], Lingxing Zheng[a], Mitali A. Tambe[c], Katherine E. Strathearn[c], Glen Acosta[a], Scott A. Hubers[c], Fang Liu[c], Seth A. Herr[a,d], Jonathan Tang[a,b], Alan Truong[b], Elwood Walls[a], Amber Pond[a], Jean-Christophe Rochet[c], Riyi Shi[a,b,*]

[a] Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, United States
[b] Weldon School of Biomedical Engineering, Purdue University, United States
[c] Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, United States
[d] Purdue University Interdisciplinary Life Sciences Program (PULSe), Purdue University, United States

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ABSTRACT

Growing evidence suggests that oxidative stress plays a critical role in neuronal destruction characteristic of Parkinson's disease (PD). However, the molecular mechanisms of oxidative stress-mediated dopaminergic cell death are far from clear. In the current investigation, we tested the hypothesis that acrolein, an oxidative stress and lipid peroxidation (LPO) product, is a key factor in the pathogenesis of PD. Using a combination of *in vitro*, *in vivo*, and cell free models, coupled with anatomical, functional, and behavioral examination, we found that acrolein was elevated in 6-OHDA-injected rats, and behavioral deficits associated with 6-OHDA could be mitigated by the application of the acrolein scavenger hydralazine, and mimicked by injection of acrolein in healthy rats. Furthermore, hydralazine alleviated neuronal cell death elicited by 6-OHDA and another PD-related toxin, rotenone, *in vitro*. We also show that acrolein can promote the aggregation of alpha-synuclein, suggesting that alpha-synuclein self-assembly, a key pathological phenomenon in human PD, could play a role in neurotoxic effects of acrolein in PD models. These studies suggest that acrolein is involved in the pathogenesis of PD, and the administration of anti-acrolein scavengers such as hydralazine could represent a novel strategy to alleviate tissue damage and motor deficits associated with this disease.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease associated with a severe movement disorder (Hirsch and Hunot, 2009; Przedborski, 2005; Rochet et al., 2012). PD results from dopamine (DA) deficiency due to degeneration of nigral DA neurons. Although the exact cause is unknown, oxidative stress has been implicated as one of the most important contributors to nigral cell death in PD (Henchcliffe and Beal, 2008; Jenner, 2003). However, despite decades of efforts, treatments that target free radicals have been largely ineffective in reducing dopaminergic cell death and delaying or alleviating motor deficits in PD (Henchcliffe and Beal, 2008; Hirsch and Hunot, 2009). Therefore, further understanding of the mechanisms of oxidative stress and identification of a novel and more effective target is highly warranted and desirable.

We have demonstrated that acrolein, an aldehyde produced by lipid peroxidation, is capable of directly damaging nerve cells and generating free radicals (Hamann et al., 2008a; Luo and Shi, 2004, 2005; Shi et al., 2002). In addition, acrolein has a much longer half-life than better-known reactive oxygen species such as the superoxide anion (O_2^-) and hydroxyl radical (·OH) (Esterbauer et al., 1991; Ghilarducci and Tjeerdema, 1995). Furthermore, evidence from our lab and others has indicated that acrolein plays a significant role in secondary oxidative stress related to spinal cord trauma (Hamann et al., 2008a; Hamann et al., 2008b; Hamann and Shi, 2009; Park et al., 2014; Shi and Luo, 2006) and multiple sclerosis (Leung et al., 2011; Tully and Shi, 2013). These findings have led us to postulate that acrolein plays a particularly damaging role through the perpetuation of oxidative stress, enhancing cellular degeneration and functional loss. Oxidative stress is a well-established pathology in PD, and therefore we hypothesize that acrolein plays a vital role in facilitating DA neuronal cell death. Furthermore, acrolein may present a novel and effective target for therapeutic interventions aimed at suppressing oxidative stress, reducing DA cell death, and alleviating motor deficits.

* Corresponding author at: Department of Basic Medical Sciences, Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, United States.
E-mail address: riyi@purdue.edu (R. Shi).

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One of the well-known toxicities of acrolein is its ability to damage proteins through adduct formation. Acrolein-bound proteins are likely to undergo profound structural changes causing both functional alteration and toxicity (Kehrer and Biswal, 2000; Shi R. et al., 2011a; Stevens and Maier, 2008). Alpha-synuclein (aSyn) is an abundant neuronal protein that is thought to play an important role in PD pathogenesis (Kalia et al., 2013). aSyn is a major component of characteristic 'Lewy body' inclusions in the brains of PD patients, and mutations in the aSyn gene are involved in some forms of familial PD (Rochet et al., 2012). From this neuropathological and genetic evidence, it is hypothesized that aSyn aggregation plays an important role in DA cell death (Recasens et al., 2014; Rochet et al., 2012; Trojanowski and Lee, 1998). Since aSyn possesses structural components that are known to be vulnerable to acrolein adduction (*e.g.* numerous lysine residues; an unfolded protein conformation) (Shamoto-Nagai et al., 2007; Weinreb et al., 1996), we speculate that acrolein-mediated structural alterations of aSyn may lead to the formation of aggregates that could contribute to neurodegeneration in PD.

The primary objective of this study was to investigate the role of acrolein in DA cell death using a combination of cellular and *in vivo* models of PD. In addition, interactions of acrolein with aSyn were investigated in cell-free, cellular, and animal models to further elucidate the role of acrolein in DA cell death. Our data suggest that acrolein contributes to the pathological changes associated with PD through a mechanism involving the formation of toxic aSyn aggregates.

2. Materials and methods

2.1. PC12 cells

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/mL penicillin, and 5 mg/mL streptomycin. The incubator was set at 5% CO_2 at 37 °C. Culture media were changed every other day, and cells were split every week. Cells were switched from DMEM-supplemented culture medium to Hank's balanced salt solution (HBSS) before they were treated with 6-OHDA (Sigma, St. Louis, MO). 6-OHDA was prepared fresh in phosphate-buffered saline (PBS) as stock solutions and diluted to the specific concentrations upon use. Hydralazine was dissolved at 30 mM, 45 mM, and 100 mM in double-distilled water as stock solutions. Hydralazine application was typically delayed for about 15 min after the application of 6-OHDA.

2.2. MES23.5 cells

The MES23.5 dopaminergic cell line is a mouse–rat hybrid. The cells were routinely propagated in Sato's N1 medium (87.5% (v/v) DMEM, glutamine (4 mM), newborn calf serum (2%, v/v), fetal bovine serum (5%, v/v), penicillin/streptomycin (1%, v/v), 15 mM HEPES (pH 7.4), and 1× SATO (50× SATO: insulin, 0.25 mg/mL; human transferrin, 0.25 mg/mL; pyruvic acid, 2.43 mg/mL; putrescine, 0.2 mg/mL; sodium selenite, 0.25 μg/mL; progesterone, 0.315 μg/mL) as described (Crawford et al., 1992).

In one set of experiments, MES23.5 cells were used to test the effects of hydralazine on 6-OHDA-mediated cytotoxicity. One group of cells were incubated with 400 μM 6-OHDA for 2 h (6-OHDA group). In the other group, cells were treated with 500 μM hydralazine after a 15-min delay following 6-OHDA exposure (6-OHDA/HZA). Cell viability was determined using either the MTT test and expressed as percent of control, or the trypan Blue assay and expressed as a percentage of cells that excluded the trypan blue dye.

In a second set of experiments, MES23.5 cells were plated in 12-well plates at a density of 50,000 to 100,000 cells per well on coverslips pretreated with poly-L-lysine (5 μg/mL) (Crawford et al., 1992; Liu et al., 2008a). After 24 h, the cells were treated with fresh media in the absence or presence of acrolein (10 μM) for 24 h. The cultures were fixed, permeabilized, and blocked as described (Liu et al., 2008b). After washing with phosphate buffered saline (PBS) (136 mM NaCl, 0.268 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), the cells were treated overnight at 4 °C with a primary mouse antibody specific for vimentin (1:500) to monitor aggresome formation. The cells were then washed with PBS and treated with a secondary anti-mouse antibody conjugated to Alexa Fluor 488 (1:1000) for 1 h at 22 °C. The cells were mounted to slides with ProLong Gold Anti-Fade mounting media containing the nuclear stain DAPI and sealed with clear nail polish. The cells were examined using a Nikon TE2000-U inverted fluorescence microscope. Previous control experiments revealed that fluorescent staining was not observed when the cells were treated with secondary antibody in the absence of primary antibody (Liu et al., 2008a; Liu et al., 2008b).

2.3. Measurement of cell viability of PC12 cells and dopaminergic cell line

2.3.1. Trypan blue cell viability assay

Trypan blue is a vital dye that is imbibed by cells after their membranes are damaged. Normally, undamaged cells exclude trypan blue, because the chromophore is negatively charged and cannot enter the cell in the absence of breaches to the membrane. All the cells excluding the dye were considered viable, whereas labeled cells were considered otherwise dying or dead. A cell suspension (0.5 mL, 1×10^6 cells/mL in HBSS) was mixed thoroughly with 0.5 mL of 0.4% trypan blue for 2 min at room temperature. With a micropipette, 10 μL of the mixture was withdrawn to fill a hemocytometer on each side. The total number of cells and viable cells were counted under the light microscope. Percentage viability was calculated as:

$$\%\ viability = \frac{viable\ \ cells}{total\ \ cells} \times 100\%$$

The percentage viability was calculated from an average of duplicate readings from both sides of the hemocytometer. Each experiment was repeated four times.

2.3.2. MTT assay

Cells were seeded in 12-well plates at 1×10^6 cells/mL in HBSS. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was reconstituted in PBS and added to each well 1 h before the termination of the experiment. After incubation, an equal volume of MTT solubilization solution was added to each well to dissolve the remaining formazan crystals. The resulting absorbance was measured spectrophotometrically (SLT, Spectra) at 550 nm, and the background absorbance at 660 nm was subtracted from these values. For each experiment, the final MTT measurement for each sample was expressed as a percentage of the control sample (no treatment).

2.4. Preparation of primary mesencephalic cultures

Primary midbrain cultures were prepared *via* dissection of E17 embryos obtained from pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN) as described previously (Liu et al., 2008a; Liu et al., 2008b; Strathearn et al., 2014). All of the procedures involving animal handling were approved by the Purdue Animal Care and Use Committee (PACUC). The mesencephalic region containing the *substantia nigra* and ventral tegmental area was isolated stereoscopically, and the cells were dissociated with trypsin (final concentration, 26 μg/mL in 0.9% [w/v] NaCl). The cells were plated in the wells of a 48-well plate (pretreated with poly-L-lysine, 5 μg/mL) at a density of 163,500 cells per well in media consisting of DMEM, 10% (v/v) fetal bovine serum, 10% (v/v) horse serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Five days after plating, the cells were treated with cytosine arabinofuranoside (20 μM, 48 h) to inhibit the growth of glial cells.