NeuroToxicology 60 (2017) 54–69

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Acute exposure to chlorpyrifos caused NADPH oxidase mediated oxidative stress and neurotoxicity in a striatal cell model of Huntington's disease

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ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form 7 February 2017
Accepted 9 March 2017
Available online 12 March 2017

Keywords:
Chlorpyrifos
Oxidative stress
Huntington's disease
Disease-toxicant interaction
Antioxidants
NADPH oxidase

ABSTRACT

We hypothesized that expression of mutant Huntingtin (HTT) would modulate the neurotoxicity of the commonly used organophosphate insecticide, chlorpyrifos (CPF), revealing cellular mechanisms underlying neurodegeneration. Using a mouse striatal cell model of HD, we report that mutant HD cells are more susceptible to CPF-induced cytotoxicity as compared to wild-type. This CPF-induced cytotoxicity caused increased production of reactive oxygen species, reduced glutathione levels, decreased superoxide dismutase activity, and increased malondialdehyde levels in mutant HD cells relative to wild-type. Furthermore, we show that co-treatment with antioxidant agents attenuated the CPF-induced ROS levels and cytotoxicity. Co-treatment with a NADPH oxidase (NOX) inhibitor, apocynin, also attenuated the CPF-induced ROS production and neurotoxicity. CPF caused increased NOX activity in mutant HD lines that was ameliorated following co-treatment with apocynin. Finally, CPF-induced neurotoxicity significantly increased the protein expression of nuclear factor erythroid 2-related factor (Nrf2) in mutant HD cells as compared to wild-type. This study is the first report of CPF-induced toxicity in HD pathophysiology and suggests that mutant HTT and CPF exhibit a disease-toxicant interaction wherein expression of mutant HTT enhances CPF-induced neurotoxicity *via* a NOX-mediated oxidative stress mechanism to cause neuronal loss in the full length HTT expressing striatal cells.

1. Introduction

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by selective loss of medium spiny neurons (MSNs) in the striatum (Imarisio et al., 2008). HD is caused by a genetic mutation in the Huntingtin gene (*HTT*) that encodes for the Huntingtin protein (HTT) (Cowan and Raymond, 2006). Despite the genetic etiology of HD, there is burgeoning evidence to suggest that environmental factors may contribute to variability in age of onset, symptoms, and progression of the disease. A kindred study revealed that 40% of variation in the age of onset in HD patients is due to the genetic mutation while the remaining 60% may be caused by unknown environmental factors and potential genetic variability mediating gene x gene interactions (Wexler et al., 2004). Identical twin HD studies have shown variability in age of onset, symptomology, and disease progression (Sudarsky et al., 1983; Anca et al., 2004; Friedman et al., 2005).

Previous studies have provided evidence to support disease-toxicant interactions in HD. For example, a recent study identified a disease-toxicant interaction in HD wherein mutant *HTT* expressing striatal cells exhibit decreased susceptibility to manganese (Mn) neurotoxicity compared to wild-type. There was also a decrease in net Mn accumulation in mutant *HTT* cells and a striatal specific decrease in Mn accumulation in HD mice following exposure to Mn (Williams et al., 2010). Principal component analysis of proteomes in the striata of wild-type and HD mice (YAC128Q) suggest that an interaction between mutant HTT and Mn may suppress proteomic phenotypes and reveal potential targets in novel treatment strategies for HD (Wegrzynowicz et al., 2012). Another study has revealed that alterations in the ataxia telangiectasia mutated tumor protein p53 (ATM-p53) signaling pathway underlies the selective alterations in Mn homeostasis in cellular models of HD (Tidball et al., 2015). HTT also mediates endocytosed iron that is required for energy production and defects in iron homeostasis and energy metabolism are both features of HD pathogenesis (Lumsden et al., 2007). Post-mortem studies have revealed

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http://dx.doi.org/10.1016/j.neuro.2017.03.004
0161-813X/

disrupted level of metals in HD brains when compared to age-matched controls (Dexter et al., 1991). These and other studies suggest that perturbation in the normal role of HTT in modulating metal homeostasis may contribute to HD pathology, further supporting the potential modulation of HD pathophysiology by environmental agents.

Despite the presence and bioaccumulation of organophosphate (OP) compounds in the environment (Ferrer, 2003), their accumulation and effect in the basal ganglia following overexposure (Bhatt et al., 1999; Müller-Vahl et al., 1999; Liu, 2007), and their established links to other neurodegenerative disorders such as Parkinson's disease (Shahar and Andraws, 2001; Kwon and Kim, 2014) that share some pathophysiological mechanisms with HD, there is scarcity of literature examining the influence of the commonly used OP compound, chlorpyrifos (CPF), in the pathophysiology of HD. CPF is an extensively used OP insecticide for crop protection against pests, golf course turf, industrial plants and vehicles, wood treatments, and to control public health pests such as mosquitoes and fire ants. The major routes of exposure for the general population are ingestion of a treated food source, contact with applications in or near residential sites and industrial means, and exposure to contaminated water (Eaton et al., 2008). The primary mechanism of CPF-induced toxicity is the irreversible inhibition of the acetylcholine-degrading enzyme, acetylcholinesterase (AChE), at cholinergic synapses, resulting in the accumulation of ACh in the body (Casida and Quistad, 2004; Costa, 2006). CPF can also disrupt non-cholinergic cellular mechanisms including oxidative stress, deranged calcium homeostasis, mitochondrial dysfunction, protein aggregation, and activation of cell death pathways (Crumpton et al., 2000; Schuh et al., 2002; Giordano et al., 2007; Binukumar et al., 2010; Katalinić et al., 2013; Androutsopoulos et al., 2013; Wang et al., 2013). OP compounds, such as CPF, can adversely affect ATP synthesis by disrupting mitochondrial membrane potential, inducing cytochrome *c* release from mitochondria into the cytosol and cause caspase-mediated apoptotic cell death (Kaur et al., 2007; Binukumar et al., 2010).

Residential use of CPF was banned in 2000 due to concerns regarding its greater effects in children as compared to adults (Eaton et al., 2008). Nevertheless, the effects caused by potential overexposure to CPF in children who reside in agricultural communities persist and remain a major human health concern (Koch et al., 2002; Arcury et al., 2007). These concerns stem in part, from the teratogenic effects of CPF that negatively impacts neurodevelopment and cause long-term cellular and behavioral effects on the nervous system (Roinestad et al., 1993; Hore et al., 2005; Morgan et al., 2005). Subsequent to exposure, CPF is metabolized in the liver to CPF-oxon and 3,5,6-trichloropyridinol (TCP) *via* cytochrome P450 enzymes (Sultatos 1994; Timchalk et al., 2007). The cytochrome P450 enzymes are also expressed in the striatum (Riedl et al., 2000). CPF can cross the blood brain barrier and accumulate in the brain following exposure and the half-life of CPF in the bloodstream is 24 h post exposure (Hayes, 1982).

Animal studies have revealed that overexposure to OP compounds, including CPF, can result in its accumulation in the basal ganglia causing extrapyramidal features that are hypothesized to be due to the pharmacological actions of higher concentrations of OP compound within the basal ganglia (Liu, 2007). The OP-induced increase in acetylcholine (ACh) concentration in the cholinergic interneurons of the striatum has the ability to stimulate efferent enkephalin-containing GABA projections to the globus pallidus externus, thus altering motor coordination (Müller-Vahl et al., 1999). A recent study demonstrated the effect of insecticides and other neurotoxins on the aggregation of mutant HTT (Deshmukh et al., 2012). Specifically, mitochondrial complex I inhibitors (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and rotenone), and complex II inhibitor (3-nitropropionic acid, a chemical model of HD), accelerate the aggregation of mutant HTT *in vitro*, although the amounts of aggregates formed varied in all cases (Deshmukh et al., 2012). Given some of the shared similarities between HD pathophysiology and the neurotoxicity of OP compounds, including oxidative stress, energy dyshomeostasis, protein aggregation, impaired degradation pathways, altered neurotransmitter synthesis and release, and metal dyshomeostasis, it is likely that overexposure to CPF may exacerbate the neurotoxic properties of mutant HTT and thus hasten the pathophysiology of HD. Exogenous treatment with antioxidants, including curcumin, ascorbic acid, and NAC is known to ameliorate CPF-induced cytotoxicity (Slotkin and Seidler, 2010) and degeneration in HD models (Acuña et al., 2013; Wright et al., 2015; Chongtham and Agrawal, 2016).

We propose that CPF and its metabolites act upon cellular targets modulated in HD and will show disease-toxicant interactions in a striatal cell model of HD. Although CPF-induced neurotoxicity is not the primary cause of HD, its mechanisms of action in tandem with the expression of mutant HTT are critical to expand our current understanding of the pathophysiology driving HD and may elucidate mechanisms of selective neurodegeneration in HD due to genetic factors, environment, or both. We hypothesized that expression of mutant HTT would enhance CPF-induced cytotoxicity *via* an oxidative stress mechanism.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Media (DMEM), Geneticin (G418), *N*-acetylcysteine (NAC), ascorbic acid, curcumin, metaphosphoric acid, superoxide dismutase (SOD) activity assay, apocynin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, nicotinamide adenine dinucleotide phosphate (NADPH), and reagents for Krebs Ringer buffer (5.6 mM glucose, 1.3 mM EDTA, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4.7 mM KCl, 120 mM NaCl, 16 mM Na_3PO_4), Sorenson's Buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5), dimethyl sulfoxide (DMSO), and radioimmunoprecipitation assay (RIPA) (50 mM Tris, 150 mM NaCl2, 0.1% SDS, 1.0% Nonidet 40) buffer were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biowest (Kansas City, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Molecular Probes by Life Technologies (Grand Island, NY, USA). CM-H_2DCFDA dye was purchased from Life Technologies (Grand Island, NY). Modified Eagles Media without phenol red was purchased from Corning Cellgro (Manassas, VA, USA). Penicillin/Streptomycin (P/S), trypsin, GlutaMax, and non-essential amino acids (NEAA) were purchased from Gibco by Molecular Probes/Life Technologies (Grand Island, NY, USA). CPF, CPF-oxon, and TCP were purchased from Chem Service (Westchester, PA, USA). Glutathione (GSH/GSSG) Detection Kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). TBARS lipid peroxidation (MDA) assay kit (ab118970) was purchased from Abcam (Cambridge, MA, USA). RNeasy Mini Kit (catalog #74104), QuantiTect Reverse Transcription Kit (catalog #2005311), and Rotor-Gene SYBR Green Kit (catalog #204074) were purchased from Qiagen (Germantown, MD, USA.).

2.2. Cell culture model

We utilized an immortalized mouse striatal cell model of HD (wild-type STHdh$^{Q7/Q7}$) and mutant STHdh$^{Q111/Q111}$) (Trettel et al., 2000) cultured in DMEM supplemented with 10% FBS, 1% P/S, 400 μg/ml G418, 1% Glutamax and 1% NEAA. Cells were passaged and/or exposed at 70–80% confluence.