Toxicology 318 (2014) 59–68

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Astrocytes protect against diazinon- and diazoxon-induced inhibition of neurite outgrowth by regulating neuronal glutathione

Daniella M. Pizzurro[a], Khoi Dao[a], Lucio G. Costa[a,b,*]

[a] *Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA*
[b] *Department of Neuroscience, University of Parma, Parma, Italy*

ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 17 January 2014
Accepted 27 January 2014
Available online 19 February 2014

Keywords:
Organophosphorus insecticide
Diazinon
Diazoxon
Neurite outgrowth
Glutathione
Glial–neuronal interactions

ABSTRACT

Evidence demonstrating that human exposure to various organophosphorus insecticides (OPs) is associated with neurobehavioral deficits in children continues to emerge. The present study focused on diazinon (DZ) and its active oxygen metabolite, diazoxon (DZO), and explored their ability to impair neurite outgrowth in rat primary hippocampal neurons as a mechanism of developmental neurotoxicity. Both DZ and DZO (0.5–10 μM) significantly inhibited neurite outgrowth in hippocampal neurons, at concentrations devoid of any cytotoxicity. These effects appeared to be mediated by oxidative stress, as they were prevented by antioxidants (melatonin, N-t-butyl-alpha-phenylnitrone, and glutathione ethyl ester). Inhibition of neurite outgrowth was observed at concentrations below those required to inhibit the catalytic activity of acetylcholinesterase. The presence of astrocytes in the culture was able to provide protection against inhibition of neurite outgrowth by DZ and DZO. Astrocytes increased neuronal glutathione (GSH) in neurons, to levels comparable to those of GSH ethyl ester. Astrocytes depleted of GSH by L-buthionine-(S,R)-sulfoximine no longer conferred protection against DZ- and DZO-induced inhibition of neurite outgrowth. The findings indicate that DZ and DZO inhibit neurite outgrowth in hippocampal neurons by mechanisms involving oxidative stress, and that these effects can be modulated by astrocytes and astrocyte-derived GSH. Oxidative stress from other chemical exposures, as well as genetic abnormalities that result in deficiencies in GSH synthesis and regulation, may render individuals more susceptible to these developmental neurotoxic effects of OPs.

1. Introduction

Diazinon (DZ) and its active metabolite diazoxon (DZO) are members of the widely used class of organophosphorus insecticides (OPs) (EPA, 2011). Children in communities in close proximity to crops where these insecticides are sprayed are exposed regularly to a variety of OPs, and may be at increased risk for adverse neurological effects. Recent studies link such exposures to various neurobehavioral deficits, such as attention deficit hyperactivity disorder and lowered I.Q. (Bouchard et al., 2010; Eskenazi et al., 2007; Rauh et al., 2011; Rohlman et al., 2011). While acute effects of OPs primarily results from acetylcholinesterase (AChE) inhibition and subsequent cholinergic overstimulation, increasing evidence suggests that these compounds can exert other non-cholinergic effects, including alterations in signal transduction, inhibition of DNA synthesis, and increases in oxidative stress (Adigun et al., 2010; Guizzetti et al., 2005; Lukaszewicz-Hussain, 2010; Slotkin et al., 2006).

Young animals are more sensitive to the acute systemic effects of OPs and to their effects on the CNS than adults (Pope and Liu, 1997; Won et al., 2001). Other studies have shown long-term effects of late gestational and neonatal exposures to OPs, with an emphasis on learning and memory (Icenogle et al., 2004; Levin et al., 2008; Roegge et al., 2006), as well as neural cell development and synaptic function (Slotkin et al., 2008). Of relevance is that such neurotoxic effects at levels appear to be independent from AChE inhibition (Rush et al., 2010; Sidiropoulou et al., 2009; Yang et al., 2008), suggesting that alternative neurotoxic mechanisms of these compounds may be involved in developmental neurotoxicity.

Several studies have pointed to oxidative stress as a potential mechanism of OP neurotoxicity (Giordano et al., 2007; Lukaszewicz-Hussain, 2010; Slotkin et al., 2005). The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) results in cellular oxidative stress. This ultimately leads

---

[*] Corresponding author at: University of Washington, Department of Environmental and Occupational Health Sciences, 4225 Roosevelt Way NE, Suite 100, Seattle, WA 98105, USA. Tel.: +1 206 543 2831; fax: +1 206 685 4696.
E-mail address: lgcosta@u.washington.edu (L.G. Costa).

http://dx.doi.org/10.1016/j.tox.2014.01.010
0300-483X/

to deleterious effects on various macromolecules, including DNA, lipids, and proteins (Valko et al., 2007). Furthermore, oxidative stress is increasingly implicated in a variety of diseases, including several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Barnham et al., 2004), as well as to neurodevelopmental disorders, including autism and schizophrenia (Chauhan et al., 2012; Do et al., 2009; Tang et al., 2013).

Cellular defenses against oxidative stress include enzymes such as superoxide dismutases, catalase, and glutathione peroxidases, as well as factors such as glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and flavonoids (Valko et al., 2007). Nevertheless, these defense systems can be overwhelmed in times of acute and/or chronic stress, rendering the cell defenseless against free radicals and oxidative species. The brain is particularly vulnerable to oxidative stress due to its high oxygen consumption, oxidizable lipid content (i.e. polyunsaturated fatty acids), as well as relatively low levels of endogenous antioxidants (Barnham et al., 2004; Matés, 2000). Levels of antioxidants in the brain differ by region and cell type. Glial cells, for example, have a higher glutathione content than neuronal cells; ascorbate, however, appears to predominate in neurons (Rice and Russo-Menna, 1997).

Glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is an abundant cellular thiol tripeptide, and one of the most prominent antioxidants in the CNS (Lu, 2013). GSH is a potent defender against ROS, due to its ability to non-enzymatically scavenge free radicals, as well as its role as a co-factor for glutathione peroxidases and glutathione transferases against reactive aldehyde and peroxide accumulation within the cell (Dringen, 2000). The dysregulation of GSH redox cycling, as well as genetic deficiencies in GSH-related enzymes have been shown to adversely affect neurodevelopment and play a role in various neurodegenerative diseases (Ballatori et al., 2009; Sian et al., 1994); furthermore, GSH has been shown to modulate neurotoxicity that results from several environmental chemicals, including OPs (Giordano et al., 2007, 2008, 2006).

The present study investigated the ability of DZ and its metabolite DZO to inhibit neurite outgrowth in primary rat hippocampal neurons and its underlying mechanisms. Results show that neuritogenesis is inhibited by OP-induced oxidative stress, and is antagonized by antioxidants and by co-culture with astrocytes, which enhance neuronal GSH content.

2. Materials and methods

2.1. Materials

Neurobasal-A medium, DMEM medium, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), GlutaMAX, anti-mouse Alexa fluor-488 secondary antibody, Hoechst 33342, 2,7′-dichlorofluorescin diacetate (H_2DCF-DA), SuperSignal West Pico Chemiluminescent Substrate (Pierce), papain, and gentamicin were from Invitrogen (Carlsbad, CA). Diazinon (DZ; 99.4%), diazinon-O-analog (diazoxon; DZO; 98%) and chlorpyrifos (CPF; 99.5%) were from Chem-Service (West Chester, PA). Poly-D-lysine, antibodies: peroxidase-conjugated anti-mouse IgG, mouse anti-beta-actin, horseradish peroxidase-conjugated anti-rabbit IgG, rabbit anti-fibronectin, rabbit anti-map-2, mouse anti-tau, goat serum, dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and N-t-butyl-alpha-phenylnitrone (PBN), glutathione ethyl-ester (GSHee), L-buthionine-(S,R)-sulfoximine (BSO), reduced L-glutathione (GSH), tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), and naphthalene dicarboxaldehyde (NDA) were from Sigma–Aldrich (St. Louis, MO). Mouse β-III-tubulin antibody was from Millipore (Billerica, MA). Melatonin was from EMD Chemicals (Rockland, MA).

2.2. Preparation of fetal rat hippocampal neurons

Hippocampal neurons from embryonic day 21 Sprague–Dawley rat fetuses were prepared as previously described (Brewer et al., 1993; Guizzetti et al., 2008; Van De Mark et al., 2009). Neurons were freshly prepared and plated on poly-D-lysine-coated glass coverslips for 2 h in Neurobasal A–FBS (10%) medium to allow attachment. After 2 h, the neurons were washed once in HBSS and the medium was replaced with astrocyte-conditioned medium (ACM) for 24 h, after which neurons were treated as indicated for 24 h.

2.3. Treatments of hippocampal neurons

After 24 h in culture, neurons were treated with varying concentrations of DZ and DZO (0, 0.1, 1, 10 μM, in DMSO) for 24 h. For experiments where antioxidants were used, neurons were pre-treated with either melatonin (200 μM), N-t-butyl-alpha-phenylnitrone (PBN; 100 μM), or glutathione ethyl-ester (2.5 mM) for 3 h prior to two washes with HBSS, replacement of ACM, and treatment with DZ or DZO.

2.4. Preparation of astrocyte-conditioned medium (ACM)

Primary cultures of cortical astrocytes were prepared as previously described (Guizzetti and Costa, 1996). After 14 days in culture (with regular medium changes), flasks of confluent astrocytes were washed twice with PBS and "serum-free" DMEM–BSA (0.1%) medium was added for 24 h. This medium was considered astrocyte-conditioned. The ACM was collected from the flasks and centrifuged at 300 × g for 10 min at room temperature to pellet any cells or debris. ACM was used as the culture medium for the rest of the incubation period for all neurite outgrowth experiments in neurons.

2.5. Astrocyte-neuron co-cultures

To assess the potential for astrocytes to protect neurons from DZ- and DZO-induced inhibition of neurite outgrowth, an indirect co-culture model was used. This model provides a way to understand astrocyte–neuronal interactions in an *in vitro* system that more accurately reflects the *in vivo* processes. Hippocampal neurons were prepared as described, and plated on glass coverslips to which four paraffin beads were previously affixed to prevent their touching the astrocyte monolayer while allowing them to share the same medium. After 2 h incubation in Neurobasal A/FBS (10%) medium to allow neurons to attach, neurons were washed twice in HBSS and ACM was added, as previously described. After 24 h, the glass coverslips containing the neurons were inverted onto 24-well plates containing astrocytes, as described by Viviani et al. (1998). This astrocyte-neuron co-culture was treated with 10 μM DZ or DZO or vehicle control (0.1% DMSO) for 24 h. In some experiments, astrocytes were previously treated with L-buthionine-(S,R)-sulfoximine (BSO; 25 μM) for 24 h to deplete GSH levels.

2.6. Measurement of cell viability

Neuron viability was measured by the MTT assay, where 50 μL of MTT reagent (5 mg/mL) was added to each well after 24 h treatment with DZ or DZO. After 3 h at 37 °C, the medium was removed and the formazan reaction product was dissolved in 250 μL DMSO. Absorbance was read at 562 nm and results were expressed as mean percentage of viable cells relative to untreated controls.

2.7. Measurement of GSH levels

Total intracellular glutathione (GSH) levels were measured as previously described (Giordano et al., 2008). Briefly, neurons were homogenized in Locke's buffer and an aliquot was taken to measure the protein concentration while a second aliquot was diluted (1:1) with 10% 5-sulfosalicylic acid (SSA). The SSA fraction was centrifuged at 13,400 × g for 5 min at 4 °C and the supernatant