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Diazinon and diazoxon impair the ability of astrocytes to foster neurite outgrowth in primary hippocampal neurons

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ABSTRACT

Evidence from *in vivo* and epidemiological studies suggests that organophosphorus insecticides (OPs) are developmental neurotoxicants, but possible underlying mechanisms are still unclear. Astrocytes are increasingly recognized for their active role in normal neuronal development. This study sought to investigate whether the widely-used OP diazinon (DZ), and its oxygen metabolite diazoxon (DZO), would affect glial–neuronal interactions as a potential mechanism of developmental neurotoxicity. Specifically, we investigated the effects of DZ and DZO on the ability of astrocytes to foster neurite outgrowth in primary hippocampal neurons. The results show that both DZ and DZO adversely affect astrocyte function, resulting in inhibited neurite outgrowth in hippocampal neurons. This effect appears to be mediated by oxidative stress, as indicated by OP-induced increased reactive oxygen species production in astrocytes and prevention of neurite outgrowth inhibition by antioxidants. The concentrations of OPs were devoid of cytotoxicity, and cause limited acetylcholinesterase inhibition in astrocytes (18 and 25% for DZ and DZO, respectively). Among astrocytic neuritogenic factors, the most important one is the extracellular matrix protein fibronectin. DZ and DZO decreased levels of fibronectin in astrocytes, and this effect was also attenuated by antioxidants. Underscoring the importance of fibronectin in this context, adding exogenous fibronectin to the co-culture system successfully prevented inhibition of neurite outgrowth caused by DZ and DZO. These results indicate that DZ and DZO increase oxidative stress in astrocytes, and this in turn modulates astrocytic fibronectin, leading to impaired neurite outgrowth in hippocampal neurons.

Introduction

There is increasing concern in the public and regulatory spheres over exposure to organophosphorus insecticides (OPs) and their ability to adversely affect neurodevelopment (Costa, 2006; Eaton et al., 2008; Eskenazi et al., 2008). The cause for concern resides in the fact that there is widespread exposure of children to OPs, both in rural and urban environments (Barr et al., 2004; Beamer et al., 2008; Eskenazi et al., 2007; Fenske et al., 2002; Lu et al., 2000; Valcke et al., 2006). Additionally, evidence from animal studies indicates that the developing nervous system may be more susceptible to the neurotoxicity of OPs than the mature nervous system (Moser et al., 1998; Moser and Padilla, 1998; Pope and Liu, 1997; Won et al., 2001). This is compounded by epidemiological studies that link early exposure to OPs and various neurobehavioral deficits in children, such as increased incidence of 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 toxicity to OPs primarily occurs as a result of acetylcholinesterase (AChE) inhibition, the mechanisms of lower-level, chronic exposures on neurodevelopment remain unclear. As reviewed by Lukaszewicz-Hussain (2010), several studies support the ability of various OPs to induce oxidative stress in humans (Ranjbar et al., 2005; Vidyasagar et al., 2004), in animal models (Jafari et al., 2012; Slotkin et al., 2005; Yilmaz et al., 2012), and in various *in vitro* models (Crumpton et al., 2000; Giordano et al., 2007; Lee et al., 2012; Slotkin and Seidler, 2009). These effects manifest in the form of altered levels and activity of different antioxidant factors, as well as increases in various markers of oxidative stress, including increased lipid peroxidation and levels of reactive oxygen species (ROS). ROS, including O_2^-, •OH, and hydrogen peroxide, are produced by a variety of enzymatic and chemical processes, many of which are an integral part of normal physiological functioning and cell signaling (Dickinson and Chang, 2011). In contrast, the overabundance and mismanagement of ROS lead to oxidative stress, which is more recently implicated in the progression of various neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease, as well as Frederick's ataxia and Amyotrophic lateral sclerosis (Barnham et al., 2004; Potashkin and Meredith, 2006). Additionally, oxidative damage and related mechanisms have been

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more recently implicated in other instances of neurodevelopmental dysfunction, such as autism spectrum disorders and schizophrenia (Do et al., 2009; Frustaci et al., 2012; Tang et al., 2013).

Underscoring the link between OP-induced oxidative stress and the susceptibility of the developing brain to these exposures, Samarawickrema et al. (2008) provide evidence of increased lipid peroxidation in fetal cord blood samples obtained from pregnant women living in a rural farming community that were exposed to OPs during crop-spraying season. These increases correlated with significantly inhibited fetal butyrylcholinesterase activity (Samarawickrema et al., 2008). The brain, and specifically the developing brain, is particularly susceptible to oxidative damage. This is due to its high oxygen consumption, high lipid content, and a relatively low amount of endogenous antioxidants (Lukaszewicz-Hussain, 2010; Matés, 2000). While these findings suggest that oxidative stress may play a role in the developmental neurotoxic mechanisms of OPs, possible consequences of such oxidative stress are for the most part unknown.

The OP diazinon (DZ) is widely used in agriculture in the U.S. and abroad, though it has been banned for residential use in the U.S. in 2004 (EPA, 2011). Those living in close proximity to crops sprayed with DZ are at risk for increased exposure and subsequent adverse health effects (ATSDR, 2008). The literature suggests that DZ and its oxygen-metabolite diazoxon (DZO) may be developmental neurotoxicants, but the mechanisms by which they exert these effects are unclear. Developmental effects are evident in studies of long-term effects of late gestational and neonatal exposures to DZ: early exposure to diazinon affected learning and memory (Levin et al., 2008; Roegge et al., 2006; Timofeeva et al., 2008), as well as neural cell development and synaptic function (Slotkin et al., 2008) in adolescent rodents. Most studies attempting to explain mechanisms of DZ and DZO neurotoxicity have been completed in cell lines (Axelrad et al., 2003; Flaskos et al., 2007; Sidiropoulou et al., 2009), primarily focused on direct damage to neurons. The present study highlights the effects of DZ and DZO on astrocyte function and their ability to foster neuronal development, using primary cell cultures of cortical astrocytes and hippocampal neurons to explore a novel mechanism of OP developmental neurotoxicity.

Previous work in our laboratory had shown that manganese and oxidants (hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)) affected astrocyte–neuronal interactions leading to impaired neuritogenesis (Giordano et al., 2009). Astrocytes are increasingly recognized as having essential roles in the function and development of the brain (Benarroch, 2005; Guizzetti et al., 2008; He and Sun, 2007). Astrocytes tend to be more resistant to oxidative stress than other neural cell types, likely due to the fact that they contain higher levels of endogenous antioxidants, such as glutathione, than neuronal cells (Giordano et al., 2006; Thorburne and Juurlink, 1996). However, oxidative stress in astrocytes, while not leading to decreases in cell viability, may alter astrocyte functions. In the present study, we investigated the potential for DZ and DZO to impair the ability of cortical astrocytes to foster neurite outgrowth in primary hippocampal neurons, by causing oxidative stress in astrocytes and negatively modulating expression of the pro-neuritogenic extracellular matrix protein fibronectin.

Materials and methods

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%) and diazinon-O-analog (diazoxon; DZO; 98%) 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) were from Sigma-Aldrich (St. Louis, MO). Protease inhibitors were from Roche Diagnostics (Indianapolis, IN). Mouse β-III-tubulin antibody was from Millipore (Billerica, MA). Melatonin was from EMD Chemicals (Rockland, MA). Purified human fibronectin (FN) was from BD Biosciences (Bedford, MA).

Preparation of fetal rat cortical astrocytes. Primary cultures of cortical astrocytes were prepared from E21Sprague–Dawley rat fetuses, as previously described (Guizzetti and Costa, 1996). Cultures were randomly checked for their purity (>95%) by immunofluorescence using an antibody against glial fibrillary acidic protein (GFAP). After at least 12 days in culture, astrocytes were plated in 24-well plates for astrocyte–neuron co-culture experiments (2×10^6 cells/well), on glass coverslips (2.5×10^5 cells/coverslip) for immunocytochemistry, or in 100 mm dishes for Western blot analysis (2×10^6 cells/dish).

Astrocyte treatments. Four days after plating, astrocytes were rinsed twice with PBS and placed in serum-free media (DMEM-BSA (0.1%)) for an additional 24 h, after which they were treated for 24 h with either DZ or DZO (both dissolved in DMSO), with H_2O_2, or with vehicle control (DMSO). DMSO concentrations in the treatment solutions never exceed 0.1%. In some experiments, astrocytes were pre-treated with the antioxidants melatonin (200 μM) or with N-t-butyl-alpha-phenylnitrone (PBN; 100 μM) for 3 h. After two washes with PBS, astrocytes were treated with either DZ or DZO for 24 h.

Measurement of cell viability. Astrocyte 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 15 min 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.

Preparation of fetal rat hippocampal neurons. Hippocampal neurons from E21 rat fetuses were prepared as previously described (Brewer et al., 1993; Guizzetti et al., 2008; VandeMark et al., 2009). For quantitative analysis of neurite outgrowth, neurons were plated on glass coverslips (2×10^4 cells/coverslip), previously coated overnight with 100 μg/mL poly-D-lysine at 37 °C.

Astrocyte–neuron co-cultures. Hippocampal neurons were prepared as described, and plated on glass coverslips to which 4 paraffin beads were previously affixed. After 1 h incubation in Neurobasal A/FBS (10%) medium to allow neurons to attach, the glass coverslips were inverted onto 24-well plates containing astrocytes, as described by Viviani et al. (1998). Rat cortical astrocytes were previously treated with vehicle control, DZ, DZO or H_2O_2 for 24 h, followed by wash-out and replacement of the medium with fresh "serum-free" medium (DMEM-BSA (0.1%)). Freshly isolated hippocampal neurons were co-cultured with these astrocytes for 48 h. This "sandwich" co-culture system prevents direct contact between neurons and astrocytes while allowing them to share the same medium. After 48 h of co-culture, the coverslips with the attached hippocampal neurons were removed from the plates containing the astrocytes, flipped over, and washed twice with warmed HBSS. The neurons were then fixed for 15 min at 37 °C with 4% paraformaldehyde (PFA) for quantitative morphological analysis. For experiments evaluating the effect of exogenous FN on DZ and DZO-induced inhibition of neurite outgrowth, 10 μg/mL FN was added to the co-culture system when the neurons were placed with the astrocytes for the 48 h incubation. Stock solutions of FN were prepared as per manufacturer's recommendations and stored in 1 mg/mL aliquots at −20 °C.