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Oxidative damage and neurodegeneration in manganese-induced neurotoxicity

Dejan Milatovic [a,*], Snjezana Zaja-Milatovic [a], Ramesh C. Gupta [b], Yingchun Yu [a], Michael Aschner [a,c]

[a] Vanderbilt University Medical Center, Department of Pediatrics/Pediatric Toxicology, 2215-B Garland Avenue, 11415 MRB IV, Nashville, TN 37232-0414, USA
[b] Murray State University, Breathitt Veterinary Center, Hopkinsville, KY, USA
[c] Pharmacology and the Kennedy Center for Research on Human Development, Nashville, TN, USA

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ABSTRACT

Exposure to excessive manganese (Mn) levels results in neurotoxicity to the extrapyramidal system and the development of Parkinson's disease (PD)-like movement disorder, referred to as manganism. Although the mechanisms by which Mn induces neuronal damage are not well defined, its neurotoxicity appears to be regulated by a number of factors, including oxidative injury, mitochondrial dysfunction and neuroinflammation. To investigate the mechanisms underlying Mn neurotoxicity, we studied the effects of Mn on reactive oxygen species (ROS) formation, changes in high-energy phosphates (HEP), neuroinflammation mediators and associated neuronal dysfunctions both *in vitro* and *in vivo*. Primary cortical neuronal cultures showed concentration-dependent alterations in biomarkers of oxidative damage, F_2-isoprostanes (F_2-IsoPs) and mitochondrial dysfunction (ATP), as early as 2 h following Mn exposure. Treatment of neurons with 500 μM Mn also resulted in time-dependent increases in the levels of the inflammatory biomarker, prostaglandin E_2 (PGE_2). *In vivo* analyses corroborated these findings, establishing that either a single or three (100 mg/kg, s.c.) Mn injections (days 1, 4 and 7) induced significant increases in F_2-IsoPs and PGE_2 in adult mouse brain 24 h following the last injection. Quantitative morphometric analyses of Golgi-impregnated striatal sections from mice exposed to single or three Mn injections revealed progressive spine degeneration and dendritic damage of medium spiny neurons (MSNs). These findings suggest that oxidative stress, mitochondrial dysfunction and neuroinflammation are underlying mechanisms in Mn-induced neurodegeneration.

Introduction

Manganese (Mn) is an essential nutrient and it functions as a critical cofactor in many key enzymes (Takeda, 2003; Aschner and Aschner, 2005). However, elevated occupational exposure to Mn poses a health risk. Elevated brain Mn concetrations are associated with occupational exposure to high levels of inhaled Mn such as in ferroalloy smelting, welding, mining, battery assembly and the manufacture of glass ceramics (Srivastava et al., 1991; Mergler et al., 1994; Bader et al., 1999; Bast-Pettersen et al., 2004; Racette et al. 2001, 2005; Bowler et al., 2007; Montes et al., 2008). Mn exposure also occurs in patients with liver disease, immature neonates with compromised liver function and individuals receiving contaminated well-water and parenteral nutrition therapy (Iinuma et al., 2003; Dobson et al., 2004). Mn exposure has been also associated with use of the fuel additive, methylcyclopentandienyl manganese tricarbonyl (MTT) (Kaiser, 2003; Bolte et al., 2004).

Excessive accumulation of Mn in the striatum, globus pallidus (GP) and the substantia nigra (SN) can result in a neurodegenerative disorder, commonly referred to as manganism. Epidemiological studies have suggested a causal relationship between elevated environmental Mn exposure and increased risk for Parkinsonian-like symptoms (Cotzias et al., 1968). Movement disorders inherent to manganism or Parkinson's disease (PD) become progressive and are irreversible, reflecting damage to neuronal structures (Aschner et al., 2007). In addition to targeting similar brain areas, PD and manganism share common mechanisms leading to dopaminergic (DAergic) neurodegeneration, namely, mitochondrial dysfunction, aberrant signal transduction, oxidative stress and the activation of cell death pathways (Dobson et al., 2004; HaMai and Bondy, 2004; Latchoumycandane et al., 2005; Kitazawa et al., 2005).

Upon entering the brain, Mn can be taken up into astrocytes and neurons. Astocytes serve as the major homeostatic regulator and storage site for Mn in the brain (Aschner et al., 1999). However, increased accumulation of Mn in astrocytes may alter release of glutamate and elicit excitatory neurotoxicity (Erikson and Aschner, 2003). Neuronal uptake of Mn involves transferrin (Suarez and Eriksson, 1993) and utilization of specific transporter system such as the dopamine transporter (DAT) (Anderson et al., 2007; Chen et al., 2006a). At the cellular level, Mn preferentially accumulates in mitochondria, where it disrupts oxidative phosphorylation and increases the generation of reactive oxygen species (ROS) (Gunter et al., 2006). Increased striatal concentrations of ascorbic acid and glutathione (GSH), antioxidants that when increased signal the presence of an elevated burden from ROS, as well as other markers

* Corresponding author. Fax: +1 615 936 4080.
E-mail address: dejan.milatovic@vanderbilt.edu (D. Milatovic).

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of oxidative stress, have been previously reported (Desole et al., 1994; Dobson et al., 2004; Erikson et al., 2007). Excessive production of ROS induces the oxidation of membrane polyunsaturated fatty acids, yielding a multitude of lipid peroxidation products. One such family of products is the F_2-isoprostanes (F_2-IsoPs), prostaglandin-like molecules produced by free radical-mediated peroxidation of arachidonic acid (AA) (Morrow and Roberts, 1999). These biomarkers of oxidative stress have been investigated in several *in vitro* and *in vivo* models, but not within the context of Mn-induced neurotoxicity *in vivo*. In addition to decrease in mitochondrial membrane potential and the depletion of high-energy phosphates, ROS generation is also associated with inflammatory responses and release of inflammatory mediators, including prostaglandins. Increasing reports demonstrate that inflammation contributes to neuronal damage and death (Liu et al., 2002; Milatovic et al., 2003, 2004). An uncontrolled or chronic inflammation response may cause irreversible tissue damage, fueling a self-propelling cycle of neuronal death. ROS generation is also associated with release of apoptogenic factors into the cytosol (Green and Reed, 1998). These interconnected pathways of oxidative stress, inflammation and apoptosis have been linked to the pathophysiology of neurodegenerative disease (Tansey et al., 2007).

Studies of postmortem brains of humans, non-human primates and rodents have indicated that Mn-induced neuronal damage is prominent in the striatum and other structures of the basal ganglia (Aschner et al., 2007; Perl and Olanow, 2007). Manganism is associated with alterations in integrity of DAergic striatal neurons and DA neurochemistry, including decreased DA transport function and/or striatal DA levels. Integrity of DAergic neurons in the substantia nigra pars compacta which are preferentially targeted in PD, is thought to be spared in Mn-induced parkinsonism (Aschner et al., 2007; Perl and Olanow, 2007). However, integrity of the striatal neurons that receive dopaminergic input has received relatively little attention. The medium spiny neurons (MSN) are the target of the dopaminergic innervation of the striatum, comprising more than 90% of striatal neurons (Deutch et al., 2007). MSN have radially projecting dendrites that are densely studded with spines, synapsing with dopamine and glutamate axons and providing the site of integration of several key inputs and outputs of the striatum (Day et al., 2006). Consequently, alterations in dendritic length and dendritic spine number may destabilize the structural basis of synaptic communication and thus compromise MSN function.

Given the shared symptoms characterizing manganism and PD, the present study was conducted to test the hypothesis that Mn neurotoxicity is associated with oxidative damage, neuroinflammation and altered integrity of DAergic striatal neurons. We have used biochemical and morphological approaches to investigate novel markers of oxidative stress and neuroinflammation and quantify synaptodendritic degeneration of striatal MSNs in mice exposed to Mn.

Materials and methods

Materials

Manganese chloride ($MnCl_2$) and ATP standards were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) with heat-inactivated horse serum, penicillin, streptomycin and cytosine arabinoside were purchased from Invitrogen (Carlsbad, CA). 15-$F_{2\alpha}$-IsoP-d4 (internal standard for F_2-IsoPs that contains four deuterium atoms), PGE_2 internal standard and prostaglandin F_2, E_2 and D_2 methyl esters were purchased from Cayman chemicals (Ann Arbor, MI).

Animals

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committees. C57Bl/6 female mice (obtained from Jackson laboratories, Bar Harbor, Main) between 6 and 8 weeks of age, were housed at 21 ± 1 °C, humidity $50 \pm 10\%$, and light/dark cycle 12 h/12 h, and had free access to pelleted food (Rodent Laboratory Chow, Purina Mills Inc., St Louis, MO, USA) and water. Mice were exposed to Mn by a single or three subcutaneous (s.c.) injections at the scruff of the neck. While the first group of mice received a single injection of 0 or 100 mg/kg Mn, a second group of mice received three identical injections of 0 or 100 mg/kg on days 1, 4 and 7. Both groups (4–6 mice in each group) were euthanized 24 h after the last injection. Cerebral hemispheres were immediately removed and processed for evaluation of F_2-IsoPs and PGE_2 or processed with Golgi impregnation for evaluation of synaptodendritic changes of striatal MSNs. The dose and route selections of Mn administration for this study are based on observations by Dodd et al. (2005), showing that the three Mn injection protocol produced 647% increase in striatal Mn levels relative to vehicle control mice.

Primary neuronal cultures

Primary cultures of cortical neurons were obtained from 17- to 18-day-old fetal Sprague–Dawley rats. Briefly, the cerebral cortex was isolated and placed into Hanks' balanced salt solution (HBSS) containing 0.125% trypsin. After removal of the meninges, the cerebral cortices were digested with bacterial neutral protease for 30 min at room temperature, followed by mechanical trituration with pipettes. Subsequently, cells were plated on glass coverslips placed in 6-well plates coated with poly-L-ornithine at a density of 6.7×10^5 cells/well. The neurons were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and F12 with penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Two days after plating, non-neuronal cell proliferation was inhibited by the addition of cytosine arabinoside (10 μM), and the next day, the culture media was changed to NeuroBasal supplemented with B27, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Experiments were carried out in 10- to 14-day-old cultures.

Mn concentrations (100 μM, 500 μM or 1 mM) in this study with primary cultures of cortical neurons were determined based on the relevant toxic Mn effect on mammalian cells as described in the literature. Weekly injections of Mn over a 3-month period (0, 2.25, 4.5, or 9 mg/kg) in monkeys have been shown to produce dose-related clinical signs, which are more severe in the higher dose ranges (Suzuki et al., 1975). While the basal ganglia represent the main target for Mn neurotoxicity, reflecting upon its preferential accumulation in this region (Dorman et al. 2006), Mn is also well-known to affect the cerebral cortex (Guilarte and Chen 2007), thus providing a rationale for examining cells derived from this brain region. Concentration-dependent neurotoxic effect of 100 μM, 500 μM or 1 mM Mn is also confirmed in our previous study with primary astrocytes cultures (Milatovic et al., 2007).

Biochemical assays

Quantitation of F_2-IsoPs. Upon completion of the experiments, primary neuronal cultures and brains were rapidly harvested, flash frozen in liquid nitrogen, and stored at −80 °C until analysis. Total F_2-IsoPs were determined with a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring as previously described (Morrow and Roberts, 1999; Milatovic et al., 2007). Total F_2-IsoPs were measured in primary neuronal cultures exposed to 100 μM, 500 μM or 1 mM of $MnCl_2$ for 2 h (exposure time with the most significant increase in F_2-IsoPs as indicated in previous studies). Briefly, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated and then subjected to chemical saponification using 15%