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Calpain activation is involved in acute manganese neurotoxicity in the rat striatum *in vivo*

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ABSTRACT

Manganese is essential for life, yet chronic exposure to this metal can cause a neurodegenerative disease named manganism that affects motor function. In the present study we have evaluated Mn neurotoxicity after its administration in the rat striatum. The participation of the calcium-dependent protease calpain and the apoptosis-related protease caspase-3, in Mn-induced cell death was monitored in the striatum and *globus pallidus*. Mn induced the activation of both proteases, although calpain activation seems to be an earlier event. Moreover, while the broad-spectrum caspase inhibitor QVD did not significantly prevent Mn-induced cell death, the specific calpain inhibitor MDL-28170 did. The role of NMDA glutamate receptors on calpain activity was also investigated; blockage of these receptors by MK-801 and memantine did not prevent calpain activation, nor Mn-induced cell death. Finally, studies in striatal homogenates suggest a direct activation of calpain by Mn ions. Altogether the present study suggests that additional mechanisms to excitotoxicity are involved in Mn-induced cell death, placing calpain as an important mediator of acute Mn neurotoxicity *in vivo*.

Introduction

Manganese (Mn) is an essential metal ion, as it is a cofactor for several enzymes, including glutamine synthetase in the brain. However, chronic exposure to Mn causes a neurodegenerative disease named "manganism", with symptoms that resemble Parkinson's disease (Olanow, 2004). Manganism is characterized by motor defects such as akinetic rigidity, dystonia and bradykinesia, as well as early impairment of speech, gait and balance. Although some of these symptoms resemble those observed for idiopathic Parkinson's disease (PD), the characteristic tremor of PD patients is less common in individuals suffering from manganism and they are not responsive to L-DOPA therapy (Olanow, 2004). However, while manganism and idiopathic Parkinson's disease display several similarities at the molecular and clinical levels (Aschner et al., 2009; Benedetto et al., 2009; Roth, 2009), these neurodegenerative diseases can be distinguished by the brain structures that are damaged in each case. Parkinson's disease is characterized by the degeneration of dopaminergic neurons in the *substantia nigra pars compacta*, the loss of dopaminergic terminals innervating the striatum, and the presence of intracytoplasmic Lewy bodies (Braak et al., 2003; Olanow, 2004). In contrast, chronic Mn exposure leads to Mn accumulation and neuronal loss in the *globus pallidus*, striatum, and *substantia nigra pars reticulata* (Aschner et al., 2009; Benedetto et al., 2009; Bonilla et al., 1982; Eriksson et al., 1987; Olanow, 2004; Olanow et al., 1996; Yamada et al., 1986).

Mn neurotoxicity has been associated with mitochondrial dysfunction (Tamm et al., 2008; Zhang et al., 2003). Mn inhibits oxidative phosphorylation and mitochondrial complex I in PC12 cells (Galvani et al., 1995); it disrupts the mitochondrial membrane potential in cultured astrocytes (Gonzalez et al., 2008; Yin et al., 2008) and striatal neurons (Malecki, 2001); and *in vivo* studies in rats have also shown that Mn intoxication leads to the inhibition of respiratory chain complexes, increases mitochondrial reactive oxygen species production and decreases monoamine oxidase activity (Zhang et al., 2003). Thus, Mn interference with oxidative phosphorylation and inhibition of mitochondrial activity could lead to a decline in ATP levels, compromising the energy metabolism of the cell.

Apoptosis has been suggested as a mechanism for Mn-neurotoxicity. DNA fragmentation and caspase activation have been observed after Mn exposure *in vitro* (Chun et al., 2001; Hirata, 2002). A mitochondrial apoptotic pathway induced by Mn has been implicated in astrocyte cortical cultures, involving cytochrome c release, caspase activation, PARP-1 cleavage and increased levels of Bax (Gonzalez et al., 2008; Yin et al., 2008), suggesting damage to glial cells. Although the induction of these apoptotic markers by Mn has not been demonstrated in the rat brain *in vivo*, the contribution of inflammation to neurodegeneration

Abbreviations: ER, endoplasmic reticulum; DEVD, Ac-Asp-Glu-Val-Asp-H (aldehyde); FJ-B, Fluoro Jade-B; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GLAST, glial glutamate–aspartate transporter; GLT-1, glutamate transporter-1; Glu, glutamate; MK-801, (+)-5-methyl-10,11,-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA, N-methyl-D-aspartate; NeuN, neuronal nuclei; QVD, Quinoline-Val-Asp-CH_2-O-Ph; SDS, sodium dodecyl sulfate.

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in manganism has been supported by the production of nitric oxide and interleukins by activated glia and microglia (Chen et al., 2006; Liu et al., 2006; Zhao et al., 2009).

Mn also alters the levels of neurotransmitters like dopamine, glutamate (Glu), and γ-aminobutyric acid (GABA) (Fitsanakis et al., 2006); and it diminishes D-aspartate uptake and expression of the glial glutamate transporters, glutamate transporter-1 (GLT-1) and glutamate–aspartate transporter (GLAST) (Erikson et al., 2007); suggesting that Mn promotes an excitotoxic mechanism of neuronal death in the striatum. Moreover, exposure to high levels of Glu in the presence of inhibitors of mitochondrial energy metabolism, make striatal neurons more susceptible to Glu neurotoxicity (Del Río et al., 2008). Thus, Mn neurotoxicity might result from the combination of the impairment of mitochondrial metabolism and altered glutamatergic neurotransmission. The N-methyl-D-aspartate (NMDA) Glu receptor subtype is mainly involved in Glu toxicity (Choi et al., 1988). Calcium influx through this receptor can activate several pathways including calcium-dependent proteases like calpain, which contribute to the degradation of cellular components and to excitotoxic death (Araújo et al., 2010; Bizat et al., 2003; Del Rio et al., 2008; Vosler et al., 2008). This type of death has been implicated in Mn neurotoxicity, based on early studies showing protection against Mn-induced striatal damage by the administration of the NMDA receptor antagonist, (+)-5-methyl-10,11,-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) (Brouillet et al., 1993).

The mechanisms of Mn neurotoxicity *in vivo* have not been fully elucidated. The main interest of the present study was to investigate, in an *in vivo* model, the participation of cell death-related proteases such as calpain and caspase-3 in Mn-induced cell death. While caspase-3 is a well-known executioner of apoptosis, calpain activity has been related to excitotoxicity (Araújo et al., 2010; Wang, 2000). The relation of calpain activity to the activation of NMDA receptors was also studied. Finally, studies in striatal homogenates were performed to evaluate the possibility of a direct activation of calpain by Mn ions.

Material and methods

Male Wistar rats (250–300 g) were used throughout the study, and were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and to the Rules for Research in Health Matters (México). The local Animal Care Committee approved all animal treatments. Animals were housed under standard conditions (12 h light cycle), with free access to food and water. All efforts were made to minimize the number of animals used and their suffering.

Mn administration

Mn-treated animals received an intrastriatal injection of $MnCl_2$ (Sigma Chemical Co, St. Louis MO, USA). In order to establish a dose of Mn that produces a lesion of an appropriate size for protection experiments, doses of 25, 50, 100 and 250 nmol were tested (Supplemental Fig. 1). An injection of 100 nmol of Mn was considered appropriate. Control animals received saline solution injections instead of Mn. Animals were anesthetized with 2.0% halothane in a 95% O_2/5% CO_2 mixture and placed on a stereotaxic frame (David Kopf Instruments) with the nose bar positioned at −3.3 as previously described (Del Río et al., 2008). Coordinates used were: −0.4 mm anterior from bregma, 2.8 mm lateral from midline, and 5.0 mm ventral from dura in order to inject in the dorsal striatum and *globus pallidus*. Rats were maintained under low anesthesia (0.5% halothane) throughout the injection, and 3 min after the injection was completed, the needle was withdrawn and the skin was sutured. Some animals were identically injected but instead of Mn they received an injection of 100 nmol of $CaCl_2$ or $MaCl_2$.

Administration of MK-801, memantine, MDL-28170 and caspase inhibitors

Rats treated with one dose of MK-801 received one ip injection of MK-801 (2 mg/kg, Tocris, Ellisville, MO, USA) 30 min before the intrastriatal injection of Mn. Rats treated with two doses of MK-801 received one ip injection of MK-801 (2 mg/kg) 30 min before the intrastriatal injection of 100 nmol of Mn, and a second ip injection of MK-801 (2 mg/kg) 30 min after the Mn administration. Animals were sacrificed 16 h after the intrastriatal injection of Mn to obtain samples for Western blot analysis, and at 24 h to obtain samples for histological evaluation. Memantine (30 mg/kg, Tocris, Ellisville, MO, USA) was ip administered 1 h before Mn intrastriatal injection and animals were sacrificed 24 h later for histological procedures.

Rats received a total of 50 mg/kg of the calpain inhibitor, MDL-28170 (Biomol, Plymouth Meeting, PA, USA), divided into three ip injections: one 20 mg/kg dose was administered 15 min before the intrastriatal injection of Mn, a second dose of 15 mg/kg 45 min after the Mn intrastriatal injection, and a third dose of 15 mg/kg 2 h after the Mn injection. Animals were either sacrificed 16 h after the intrastriatal administration of Mn for Western blot analysis, or at 24 h for histological evaluation.

Two different groups of animals received an intrastriatal co-injection of Mn and either the caspase-3 inhibitor, DEVD (Ac-Asp-Glu-Val-Asp-CHO, Peptide Institute, Inc. Osaka, Japan) Peptide Institute, Inc. Osaka, Japan), or the general caspase inhibitor QVD (Quinoline-Val-Asp-CH_2-O-Ph, Enzyme System Products, Ohio, USA). Stock solutions of DEVD (10 mM) or QVD (20 mM) were prepared in DMSO and an aliquot of each inhibitor was mixed with the Mn stock solution to reach a final concentration of 2.5 mM. One μl of this solution was intrastriatally injected as described above. Brains were extracted either 8 or 16 h after the intrastriatal injection, and tissues were processed for Western blot analysis. An additional series of animals was co-injected with Mn and QVD and brains were processed for histological analysis 24 h after the intrastriatal injection.

Histological evaluation

To investigate the time-course of the development of the Mn-induced lesion induced, brains were extracted under deep anesthesia at different times after Mn injection (4, 8 and 24 h). For protection experiments, animals were sacrificed 24 h after the different treatments. Rats were anesthetized with sodium pentobarbital anesthesia and transcardially perfused with 200 ml 0.9% saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and left in fixative for additional 24 h, then transferred successively to 20% and 30% sucrose (24 h each). 40 μm coronal sections were cut in a cryostat and stained with Cresyl Violet. Lesion size was calculated by examination of all brain sections where neuronal damage was evident in each experimental animal. Tissue was considered damaged when intensively stained pyknotic nuclei were present and only few or none normal appearing cells were visible. Damaged area in each tissue section was manually delineated and measured with the aid of an image analyzer (NIH Macintosh Image 1.6). The lesion volume was calculated by adding the measured areas in all sections, and multiplying the sum by the distance between the first and the last section, where damage was visible as previously reported (Del Rio and Massieu, 2008). Results are expressed as mean ± S.E.M. of the lesion volume per each animal group.

Fluoro Jade-B and immunohistochemistry experiments

Fluoro Jade-B (FJ-B, Chemicon Temecula, CA, USA) staining was performed in animals sacrificed 4 h after the intra-striatal injection of Mn. Briefly, slide-mounted coronal brain sections (20 μm) were dried at 50 °C for 30 min, ethanol solution (80% and 70%) was added and incubated for 5 and 2 min respectively, the slides were washed and covered with potassium permanganate 0.06% for 10 min, immediately washed