ELSEVIER

Brain Research Bulletin 74 (2007) 406–415

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

Research report

Manganese intoxication decreases the expression of manganoproteins in the rat basal ganglia: An immunohistochemical study

M. Morello [a], P. Zatta [b], P. Zambenedetti [c], A. Martorana [a], V. D'Angelo [a], G. Melchiorri [a], G. Bernardi [a,d], G. Sancesario [a,d,*]

[a] *Department of Neuroscience, University of Rome Tor Vergata, Rome, Italy*
[b] *Department of Biology, University of Padua, Italy*
[c] *Anatomopathology Division and Brain Bank, Dolo General Hospital, Dolo, Venice, Italy*
[d] *Santa Lucia Foundation, Rome, Italy*

Received 13 February 2007; received in revised form 12 June 2007; accepted 12 July 2007
Available online 2 August 2007

Abstract

Manganese (Mn) is a cofactor for some metalloprotein enzymes, including Mn-superoxide dismutase (Mn-SOD), a mitochondrial enzyme predominantly localized in neurons, and glutamine synthetase (GS), which is selectively expressed in astroglial cells. The detoxifying effects of GS and Mn-SOD in the brain, involve catabolizing glutamate and scavenging superoxide anions, respectively. Mn intoxication is characterized by impaired function of the basal ganglia. However, it is unclear whether regional central nervous system expression of manganoproteins is also affected. Here, we use immunocytochemistry in the adult rat brain, to examine whether Mn overload selectively affects the expression of GS, Mn-SOD, Cu/Zn-SOD, another component of the SOD family, and glial fibrillary acid protein (GFAP), a specific marker of astrocytes. After chronic Mn overload in drinking water for 13 weeks, we found that the number and immunostaining intensity of GS- and Mn-SOD-positive cells was significantly decreased in the striatum and globus pallidus, but not in the cerebral frontal cortex. In addition, we found that GS enzymatic activity was decreased in the strio-pallidal regions but not in the cerebral cortex of Mn-treated animals. In contrast, Cu/Zn-SOD- and GFAP-immunoreactivity was unchanged in both the cerebral cortex and basal ganglia of Mn-treated rats. Thus, we conclude that in response to chronic Mn overload, a down-regulation of some manganoproteins occurs in neurons and astrocytes of the striatum and globus pallidus, probably reflecting the vulnerability of these regions to Mn toxicity.

Keywords: Glutamine synthetase; Mn-SOD; Cu/Zn-SOD; Astrocytes; Globus pallidus; Striatum; Cerebral cortex; Cytochemistry; Superoxide; Glutamate toxicity

1. Introduction

Manganese (Mn) is an essential trace element in plant and animal life [11,20]. Although, it is known to be potentially toxic to the central nervous system (CNS) [1,5], its mechanism of action is complex and controversial. Mn toxicity is an occupational health hazard in miners and other workers [12,13,41]. Mn has also been identified as a nutritional contaminant in patients receiving long-term parenteral nutrition [14,21], and as an endogenous neurotoxin in patients with chronic liver failure [27]. Independent of the route of absorption, Mn overload acts as a toxicant to the brain, resulting in neurological disorders that are characterized by early psychotic symptoms and later on by irreversible parkinsonism, a syndrome known as manganism [5,12,13,35,42]. Unlike Parkinson's disease, in which the neurodegeneration primarily occurs in the substantia nigra pars compacta, Mn intoxication in humans and animal models results in prominent neuronal loss and gliosis in the globus pallidus and the caudate putamen [42,43,58].

In the past, research focused on understanding the mechanism(s) of Mn neurotoxicity has relied on the identification of a wide spectrum of non-physiological functions gained by a high concentration of Mn in the brain. Early studies hypothesized that Mn toxicity invokes enhanced auto-oxidation of dopamine [1,2,25]. It has also been suggested that the oxidation state of Mn is an important factor that contributes to its cytotoxicity [11].

* Corresponding author at: Department of Neuroscience, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy. Tel.: +39 06 20903013; fax: +39 06 72596022.
E-mail address: sancesario@med.uniroma2.it (G. Sancesario).

0361-9230/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2007.07.011

Neurodegeneration in the striatum may be linked to a cascade of oxidative damage related to the ease with which Mn can be readily oxidized from the 2+ to the 3+ oxidation state [1,2,26]. Since Mn preferentially accumulates in the mitochondria of basal ganglia, it has been suggested that the mitochondria are target organelles for Mn toxicity [31,38]. In the mitochondria, Mn can disrupt calcium homeostasis and other mitochondrial functions [24,31,34].

Although a wide range of heterogeneous biochemical effects may be linked to Mn toxicity, few studies have investigated whether Mn intoxication affects the homeostasis of the manganoproteins, and their functions and distribution in the brain [18,53]. Mn is an essential cofactor for certain metalloenzymes or manganoproteins, involved in nitrogen and oxygen metabolism [11,52]. Indeed, Mn is specifically required for maximal catalytic activity in many cases. Within the CNS, Mn is a cofactor for manganoproteins such as glutamine synthetase (GS) and superoxide dismutase (Mn-SOD) [2]. GS, selectively expressed in astroglial cells [60], catalyzes the conversion of glutamate to glutamine, thereby preventing an increase in extracellular glutamate levels and glutamate-dependent overexcitation [11,54]. Region-selective distribution of GS has been associated with areas rich in glutamate innervation [40,51]. Mn-SOD is a mitochondrial enzyme that is predominantly localized to neurons [32]. It specifically regulates and detoxifies superoxide (O_2^-), an extremely powerful oxidant and by-product of cellular metabolism.

It is possible that the expression and activity of manganoproteins may be regulated by changes in cellular Mn levels under physiological and pathological conditions. Here, we used immunocytochemistry to examine whether chronic Mn overload affected the expression of GS and Mn-SOD in the rat strio-pallidal complex and cerebral cortex, representing respectively Mn-vulnerable and Mn-resistant brain regions in human and nonhuman primates [39,41,59]. We also examined whether manganoproteins were more sensitive to Mn overload by analyzing the immunocytochemical expression of: a) copper/zinc superoxide dismutase (Cu/Zn-SOD), another component of the SOD family, that is present in neurons and in astrocytes [32]; and b) the glial fibrillary acid protein (GFAP), considered to be a sensitive marker of astroglial function. Finally, we evaluated whether chronic Mn overload affected GS enzymatic activity, since GS is reportedly very sensitive to changes in Mn concentrations [54]. Here, we report that an excess of Mn can impair some biological processes such as expression and activity of manganoproteins, that are normally regulated by Mn trace levels in the brain.

2. Experimental procedure

2.1. Animal protocols

Forty male Wistar rats (Charles River, Como, Italy), weighing 85–100 g at the start of the experiment, were randomly divided into two groups and housed in stainless steel cages under stable conditions of humidity (60 ± 5%) and temperature (22 ± 2 °C). They were fed on a standard pellet diet (RF18: Morini, Bologna, Italy), containing Mn (48 mg/kg), and were maintained on a 12 h light–dark cycle (light on from 6 a.m. to 6 p.m.; light off from 6 p.m. to 6 a.m.). The experimental protocols conformed to the guidelines of the European Union Council (86/609/EU) and were approved by the Institutional Animal Care and Use Committee of the University of Rome Tor Vergata.

Chronic Mn intoxication was induced *per os*. Briefly, rats had free access to either normal drinking water, or to a solution of $MnCl_2 \cdot 4H_2O$ (Sigma, Milan, Italy) in drinking water (20 mg/ml corresponding to about 100 mM of Mn^{2+}) [8,30,48]. The average consumption of food (11–15 g/100 g body weight) and drinking water (10–12 ml/100 g body weight) were similar at different time intervals in the two groups during the course of the experiment, as was the average body weight (300–350 g) before sacrifice. The treatment lasted for 13 weeks, and then the rats were killed for chemical, biochemical or morphological studies.

For morphological analysis, controls ($n = 10$) and Mn-exposed rats ($n = 10$) were deeply anaesthetized with chloral hydrate (400 mg/kg i.p.), followed by transcardial perfusion with the aid of a peristaltic pump (Minipuls 3®, Gilson, Middleton, USA). Initial perfusion consisted of 50 ml of saline and heparin (1%) at room temperature, followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4), at standard flow rate of 12–16 ml/min, and at a maximum pressure of 0.5 MPa. The brains were removed, postfixed in the 4% paraformaldehyde solution for 2 h at 4 °C, then stored in PB overnight. The brains were transferred in PB with 30% sucrose for 48 h, frozen and sectioned in a cryostat (Cryostat Leitz-Leica 1720, Wetzlar, Germany) at 40-μm thickness.

For chemical and biochemical studies, the animals were killed by decapitation. The brain was rapidly removed, rinsed with cold physiological saline to remove any excess blood, then blotted dry with filter paper. The striatum, the globus pallidus, and the sensori-motor cortex were dissected out, and the tissues were either dehydrated overnight in the oven at 100 °C or immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Measurement of Mn levels in brain

Samples of the different brain regions were digested by addition of four volumes of 65% HNO_3 for one week at room temperature. The Mn content was determined in five Mn-treated and five control rats by an atomic absorption spectrophotometer (Perkin-Elmer, 2100) equipped with a graphite furnace with platform and a hollow cathode Mn lamp (absorption at 279.5 nm). The Mn standard used for atomic absorption was purchased from Carlo Erba (Milan, Italy). Palladium (0.005 mg) and $Mg(NO_3)_2$ (0.003 mg) were used as matrix modifiers. An atomization temperature equal to 1900 °C was reached. Data were expressed as μg/g dry weight of brain tissue, and Student's t test was used to evaluate significant differences in the Mn content between control and Mn-treated animals.

2.3. Measurement of GS activity

GS activity was determined in tissue homogenates by measuring the γ-glutamyl hydroxamate formation in five Mn-treated and five control rats. The specific synthetase enzymatic activity in the "transferase" reactions was calculated in nM of γ-glutamyl hydroxamate formed per mg of total protein/min. as described previously [6]. Briefly, the assay reaction mixture (Tris-HCl buffer solution 0.1 M, pH 6.8) contained the following: 50 mM imidazole–HCl, 50 mM NH_2OH, 100 mM L-glutamine, 0.5 mM $MnCl_2$, 0.2 mM ADP and 25 mM KH_2AsO_4. After 30 min incubation at 37 °C, an equal volume of stop solution, consisting of 0.37 M $FeCl_3$, 0.3 M trichloroacetic acid and 0.6 M HCl, was added. The absorbance of the solution obtained was measured spectrophotometrically at 505 nm (Agilent 8453). A solution of γ-glutamyl hydroxamate was used as a standard. A protein assay was performed on an aliquot of the homogenate, using the procedure described by Lowry et al. [33]. All chemicals were purchased from Sigma (Milan, Italy). Student's *t*-test was used to evaluate significant differences in the GS activity between control and Mn-treated animals.

2.4. Histology, GS, Mn-SOD, Cu/Zn-SOD and GFAP immunoreactivities

Coronal brain sections including the sensorimotor cerebral cortex, the caudate-putamen and globus pallidus (1.7 mm anterior to 1.4 mm posterior to