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Manganese is toxic to spiral ganglion neurons and hair cells in vitro

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ABSTRACT

Occupational exposure to high atmospheric levels of Mn produces a severe and debilitating disorder known as manganism characterized by extrapyramidal disturbances similar to that seen in Parkinson's disease. Epidemiological and case studies suggest that persistent exposures to Mn may have deleterious effects on other organs including the auditory system and hearing. Mn accumulates in the inner ear following acute exposure raising the possibility that it can damage the sensory hair cells that convert sound into neural activity or spiral ganglion neurons (SGN) that transmit acoustic information from the hair cells to the brain via the auditory nerve. In this paper we demonstrate for first time that Mn causes significant damage to the sensory hair cells, peripheral auditory nerve fibers (ANF) and SGN in cochlear organotypic cultures isolated from postnatal day three rats. The peripheral ANF that make synaptic contact with the sensory hair cells were particularly vulnerable to Mn toxicity; damage occurred at concentrations as low 0.01 mM and increased with dose and duration of Mn exposure. Sensory hair cells, in contrast, were slightly more resistant to Mn toxicity than the ANF. Mn induced an atypical pattern of sensory cell damage; Mn was more toxic to inner hair cells (IHC) than outer hair cells (OHC) and in addition, IHC loss was relatively uniform along the length of the cochlea. Mn also caused significant loss and shrinkage of SGN soma. These findings are the first to demonstrate that Mn can produce severe lesions to both neurons and hair cells in the postnatal inner ear.

1. Introduction

The biological requirement of Mn as an essential trace mineral for normal growth and development was first recognized almost 80 years ago (Kemmerer et al., 1931; Orent and McCollum, 1931). As an essential nutrient, Mn is necessary for normal homeostatic processes controlling reproduction, formation of connective tissue and bone, carbohydrate and lipid metabolism and brain function (Bourre, 2006; Keen et al., 1999). Mn deficiency during fetal development can result in neurological and behavioral deficits as well as abnormal growth of a variety of systems in the body (Hurley, 1981; Strause et al., 1986). Mn deficiency, however, in the adult population is essentially nonexistent because of the abundant supply of Mn in our normal diet. In contrast, Mn intoxication caused by prolonged exposures produces a severe and debilitating disorder known as manganism (Krieger et al., 1995; Pomier-Layrargues et al., 1995). The most prominent and severe disabilities associated with excess exposure to Mn include a distinct extra pyramidal syndrome which resembles the dystonic movements associated with Parkinson's disease (Huang et al., 1993; Olanow et al., 1996; Pal et al., 1999). Manganism is generally considered to be an occupational disorder being observed most often in individuals whose profession involves protracted contact with high atmospheric levels of Mn such as welders, Mn miners and individuals employed in ferroalloy processing. Patients with chronic hepatic failure also display elevated serum and brain levels of Mn and exhibit many of the behavioral deficits and neurodegenerative features observed in occupationally exposed workers primarily because the liver is the major organ responsible for its elimination from the body (Burkhard et al., 2003; Hauser and Zesiewicz, 1996; Hauser et al., 1994; Krieger et al., 1995; Pomier-Layrargues et al., 1995).

The classical symptoms of manganism were originally described almost 170 years ago by Couper (Couper, 1837; Lucchini et al., 2009; Santamaria and Sulsky, 2010) in a man using a grinding wheel composed of the black oxides of manganese. Although sporadic reports of Mn toxicity appeared in the literature within the first half of the previous century, it has only been in the last several decades that significant progress has been made in understanding the mechanisms of Mn cytotoxicity. Manganism is considered an occupational disorder largely restricted to workers in industrial environments where the Mn atmospheric levels exceed the requisite threshold limit value (TLV). Major concerns about Mn exposure in the general population, however, were recently raised with the proposed use of methylcyclopenta-

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dienyl manganese tricarbonyl (MMT) as a fuel additive to boost octane ratings in gasoline.

The preponderance of clinical and basic research concerning the toxic actions of Mn has primarily focused on central nervous system (CNS) effects with almost complete indifference to other pernicious manifestations which may be equally irreversible though considerably less perceptible. What is becoming evident is that chronic exposure to Mn may also have harmful effects to other tissues in the body including the auditory system. For example, several reports in the literature have described hearing deficits both in welders and alloy worker who are normally exposed to chronic high levels of Mn and in individuals exposed simultaneously to noise and Mn (Bouchard et al., 2008; Josephs et al., 2005; Khalkova and Kostadinova, 1986; Korczynski, 2000). However, because of the confounding effects of workplace and recreational noise, it is uncertain if the hearing loss is caused by Mn exposure, noise exposure or the combined effects of manganese and noise (Nikolov, 1974). Based on the limited findings in the literature, it is unclear as to whether Mn alone is actually responsible for the hearing deficits reported or if hearing loss is due to other confounding factors such as noise exposure. Given the recent report demonstrating that Mn accumulates in the inner ear (Ma et al., 2008), it is reasonable to hypothesize that it has the potential to exert its cytotoxic effects on the sensory hair cells, neurons or supporting cells which in turn would be expected to result in significant hearing loss. To explore its potential toxic effects on the inner ear, we treated postnatal cochlear organotypic cultures with varying doses of Mn.

2. Materials and methods

2.1. Cochlear organotypic cultures

Cochlear organotypic cultures were prepared from postnatal day 3 SASCO Sprague–Dawley rats as described previously (Corbacella et al., 2004; Ding et al., 2002; Wei et al., 2010). In brief, the cochlea was removed and the organ of Corti and SGN were transferred onto rat tail type I collagen gel in basal medium Eagle containing 2% sodium carbonate. A 15-μL drop of the collagen solution was placed on the surface of a 35 mm culture dish and allowed to gel for approximately 30 min. Afterwards, 1.3 ml of culture medium (0.01 g/ml bovine serum albumin, 1% Serum-Free Supplement [Sigma I-1884], 2.4% of 20% glucose, 0.2% penicillin G, 1% BSA, 2 mM glutamine, 95.4% of 1× BME) was added to the dish. The cultures were maintained in an incubator at 37 °C and 5% CO_2 overnight. On the following day, fresh medium was added alone or containing various concentrations of Mn.

2.2. Mn chloride treatments

$MnCl_2$ stock solution was freshly made at a stock concentration of 10 mM in serum-free medium and diluted to final concentrations varying from 0.01 to 5.0 mM. Cochlear explants (n = 6/group) were incubated in the presence or absence of Mn in 5% CO_2 and 37 °C in humidified atmosphere from 24 to 96 h.

2.3. Histological evaluation

Cochlear explants were fixed for 2 h in 4% formalin and subsequently washed with 0.1 M phosphate buffered saline (PBS). As described in our previous publications, the specimens were immunolabeled with a primary monoclonal antibody against neuronal class III β-tubulin (Covance, MMS-435P) which was detected using a secondary antibody labeled with Cy3 (goat anti-mouse IgG, Jackson ImmunoResearch; #115-165-206) (Ding et al., 2002; Lanzoni et al., 2005; McFadden et al., 2003; Qi et al., 2008). To visualize F-actin that is heavily expressed in the cuticular plate and stereocilia bundles of hair cells, specimens were labeled with phalloidin conjugated Alexa Fluor 488 (Invitrogen A12379, diluted by 1:200). After rinsing with 0.1 M PBS, specimens were mounted on glass slides in glycerin, coverslipped and examined using a confocal microscope (Zeiss LSM-510 meta, step size 0.5 μm per slice) using appropriate filters to detect the fluorescence of Cy3 labeled product in nerve fibers and spiral ganglion neurons (SGN) (excitation 550 nm, emission 570 nm) and green fluorescence of Alexa 488-labeled phalloidin (excitation 488 nm, emission 520 nm) that labels the bundles of stereocilia and the cuticular plate of the hair cells. Confocal images were stored on disk and processed using Confocal Assistant, ImageJ and Adobe Photoshop 5.5 software.

2.4. Nerve fibers

The fascicles of auditory nerve fiber (ANF) bundles projecting out from the SGN to the organ of Corti were counted across the width (120 μm) of the field of view of the microscope at a magnification of 630×. All the fibers were counted in the same region in the middle of the cochlear culture. Five organotypic cultures were examined for each experimental condition. Data were analyzed using a one-way ANOVA followed by Newman–Keuls post hoc analyses (GraphPad Prism 5 software).

Cochlear hair cells were observed under a fluorescent microscope with the appropriate filter to visualize the stereocilia and cuticular plate of hair cells that are intensely labeled by Alexa 488-labeled phalloidin. A hair cell was counted as missing if the stereocilia were missing or severely damaged. The three rows of outer hair cells (OHC) and single row of inner hair cells (IHC) were counted along the entire length of cochlea from apex to base. A cochleogram was used to determine the percent of IHC and OHC as a function of percent distance from the apex to the base. Using custom cochleogram software and laboratory norms from control animals, the average (n = 5/condition) percentage of hair cells missing was plotted as a function of percent distance from the apex of the cochlea for each experimental group as previously described (Wei et al., 2010).

3. Results

3.1. Mn damages hair cells and nerve fibers

Studies were performed to determine the effect of Mn concentration and exposure time on ANF viability. For these experiments, cochlear organotypic cultures were treated for 24, 48 or 96 h with doses of Mn ranging from 0.01 to 5 mM. Fig. 1 shows the condition of the IHC, OHC, ANF and SGN in a typical control specimen cultured for 96 h without Mn treatment (0 mM). The actin in the stereocilia bundle and cuticular plate of the OHC and IHC is heavily labeled with phalloidin-Alexa Fluor 488. The three rows of OHC and single row of IHC are arranged in orderly rows that spiral from the base to the apex of the cochlea. The SGN, ANF and nerve terminals (NT) are intensely labeled with β-tubulin. The peripheral ANF of the SGN radiate outward towards the IHC and OHC and form NT on the hair cells. The hair cells, ANF, NT and SGN in untreated (0 mM) controls appeared normal and showed no obvious signs of pathology after being cultured for 96 h or less as illustrated in Fig. 1. The normal appearance of these untreated control cultures is consistent with our previous results (Wei et al., 2010).

The photomicrographs in Fig. 2 illustrate the degenerative changes after 24 h exposure to Mn. Doses of Mn ranging from 0.01 to 1 mM (Fig. 2A–E) had little effect on the ANF as they radiate out towards the single row of IHC and three rows of OHC. The ANF