Manganese Induces the Mitochondrial Permeability Transition in Cultured Astrocytes

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Manganese is known to cause central nervous system injury leading to parkinsonism and to contribute to the pathogenesis of hepatic encephalopathy. Although mechanisms of manganese neurotoxicity are not completely understood, chronic exposure of various cell types to manganese has shown oxidative stress and mitochondrial energy failure, factors that are often implicated in the induction of the mitochondrial permeability transition (MPT). In this study, we examined whether exposure of cultured neurons and astrocytes to manganese induces the MPT. Cells were treated with manganese acetate (10–100 μM), and the MPT was assessed by changes in the mitochondrial membrane potential and in mitochondrial calcein fluorescence. In astrocytes, manganese caused a dissipation of the mitochondrial membrane potential and decreased the mitochondrial calcein fluorescence in a concentration- and time-dependent manner. These changes were completely blocked by pretreatment with cyclosporin A, consistent with induction of the MPT. On the other hand, similarly treated cultured cortical neurons had a delayed or reduced MPT as compared with astrocytes. The manganese-induced MPT in astrocytes was blocked by pretreatment with antioxidants, suggesting the potential involvement of oxidative stress in this process. Induction of the MPT by manganese and associated mitochondrial dysfunction in astrocytes may represent key mechanisms in manganese neurotoxicity.

Manganese is an essential element and an integral component of key enzymes such as glutamine synthetase and mitochondrial superoxide dismutase (Mn-SOD)1 (1). However, excess deposition of manganese in the central nervous system often leads to neurological abnormalities, including manganese toxicity. Manganese is an occupational health problem in workers employed in welding industries; it has also been implicated in the mechanism of hepatic encephalopathy, a neurological condition associated with chronic liver failure (5, 6). The mechanisms of manganese neurotoxicity are not completely understood. Several reports propose that manganese neurotoxicity is mainly associated with mitochondrial dysfunction (7), leading to decreased oxidative phosphorylation (8). Manganese has also been shown to induce oxidative stress (9–11).

Oxidative stress is considered a major factor in the induction of the mitochondrial permeability transition (MPT) (12, 13). The MPT is a Ca2+-dependent process often associated with various other factors (14). The MPT is characterized by opening of the permeability transition pore in the inner mitochondrial membrane, resulting in increased permeability of this membrane to protons, ions, and other solutes (>1500 Da). This increased permeability leads to a collapse of the mitochondrial inner membrane potential (∆Ψm), colloid osmotic swelling of the mitochondrial matrix, defective oxidative phosphorylation, and cessation of ATP synthesis, ultimately resulting in mitochondrial failure (15). The MPT is characteristically blocked by cyclosporin A (CsA) (16).

Because manganese toxicity is associated with oxidative stress, a major inducer of the MPT, we examined the possible role of the MPT in the mechanism of manganese neurotoxicity. The MPT was assessed in cultured astrocytes and neurons after exposure to different concentrations of manganese acetate for variable time points.

EXPERIMENTAL PROCEDURES

Materials—TMRE (tetr methylrhodamine ethyl ester) and calcine/AM were obtained from Molecular Probes (Eugene, OR). All other chemicals were from Sigma and were of analytical grade.

Astrocyte Cultures—Primary cultures of astrocytes were prepared from cerebral cortices of 1–2-day-old rats. Briefly, cortices were freed of meninges, minced and dissociated by trituration, passed through sterile nylon sieves, and placed in Dulbecco’s modified Eagle’s medium (DMEM) containing penicillin, streptomycin, and 15% fetal bovine serum. Approximately 0.5 × 105 cells/ml were seeded in 35-mm culture plates and maintained at 37 °C in an incubator equilibrated with 5% CO2 and 95% air. Cultures consisted of 95–99% astrocytes based on immunohistochemical staining with glial fibrillary acidic protein. 3–5-week-old cells were used for experiments. For each experiment, 6–8 culture plates were selected randomly from at least two different seedling batches. Astrocyte cultures were exposed to different concentrations of manganese acetate (10–100 μM) for 3–48 h.

Neuronal Cultures—Cortical neuronal cultures were prepared by a modification of the method of Hertz and Martz (17). Briefly, cerebral cortices were removed from 16–18-day-old rat fetuses and placed in high glucose DMEM (Invitrogen) containing 25 mM KCl and 20% horse serum. The tissue was minced and mechanically dissociated with a pipette. Approximately 1–2 × 105 cells/ml were seeded onto poly-L-
lysine-coated 35-mm culture dishes. To prevent proliferation of astrocytes, cytosine arabinoside (10 μM) was added for 24 h to the culture medium 48 h after seeding. These cultures consisted of at least 95% neurons as determined by immunohistochemical staining for neurofilament protein; the remaining cells were astrocytes. Experiments were performed on cultures that were 6–8-days-old.

Measurement of ΔΨm—The ΔΨm was measured with the fluorescent dye TMRE as previously described (18, 19). At the end of treatment, the culture medium was removed (duplicate plates per experiment; repeated two times), and the cells were loaded with TMRE in DMEM without phenol red and serum at a final concentration of 50 nM for 20 min. The cells were rinsed with DMEM and examined with a Nikon Diaphot inverted fluorescent microscope equipped with multivariant fluorescent filters. The fluorescence emission was recorded at 590 nm. At least 20 image fields with a similar degree of cell density in each experimental group were randomly captured with a cooled digital camera and the fluorescent intensities analyzed using the NIH software (Scion Incorporation, Frederick, MD). In each image field, the total number of pixels was quantified on a gray scale (0–255 counts). The mean pixel value in each image field was obtained and expressed as mean ± S.E. of the total number of mean pixel values in each experimental group. The fluorescent intensities were expressed as percent fluorescence change over control. To ensure that the total number of cells in each field was indeed similar, pilot studies were carried out in which nuclei were counterstained with Hoechst 33258 (2 cells in each field was indeed similar, pilot studies were carried out in fluorescence change over control. To ensure that the total number of mental group. The fluorescent intensities were expressed as percent.

To rule out the possibility that manganese directly quenches TMRE fluorescence (i.e. independent of the MPT), we incubated medium containing TMRE (50 nM) with 10 μM manganese acetate for 30 min at 37 °C. Fluorescence intensity was measured spectrofluorometrically at an excitation λ 485 and emission λ 590. Manganese had no effect on medium TMRE fluorescence (data not shown).

Mitochondrial Permeability Using Calcein Fluorescence—To demonstrate the induction of the MPT, calcein fluorescence studies were carried out as previously described (18, 20) following the method of Petronilli et al. (21). This method allows one to directly visualize permeability changes in mitochondria in situ. Calcein/acetoxymethyl ester enters the cells and becomes fluorescent upon de-esterification. Co-loading of cells with cobalt chloride quenches the fluorescence in the cell, except in mitochondria, because cobalt is impermeable across mitochondrial membranes. However, during induction of the MPT, cobalt enters mitochondria and is able to quench the mitochondrial calcein fluorescence. At the end of manganese treatment (24 h), cultures were washed three to four times in Hanks balanced salt solution (HBSS; 144 mM NaCl, 10 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, 5 mM KCl, and 10 mM glucose, pH 7.4). Cells were incubated at 37 °C in fresh HBSS containing calcein/acetoxymethyl ester (1 μM) and cobalt chloride (1 mM) for 20 min. Following cobalt quenching, cultures were washed with HBSS, and images were collected within 30 min as described above. We have validated this method in cultured astrocytes by the addition of 500 μM CoCl2 along with the calcium ionophore A23187 (3 μM) to cultures (in HBSS) previously loaded with calcein and Co2+. The addition of calcium caused a significant decrease in the mitochondrial calcein fluorescence in a time-dependent manner (data not shown), consistent with the earlier report by Petronilli et al. (21).

To confirm that manganese has no effect on the loading of calcein into astrocytes, which otherwise would confound our results, astrocytes were incubated for 20 min in HBSS containing 1 μM calcein/acetoxymethyl ester but without CoCl2 in the presence or absence of manganese (100 μM). Images of normal and manganese-treated astrocytes were captured and subjected to fluorescence quantitation as described above. The results showed no difference in the fluorescent intensity in astrocytes in the presence or absence of manganese, indicating that manganese had no effect on calcein loading into astrocytes (data not shown).

Statistical Analysis—Data are presented as means ± S.E. The data were analyzed by analysis of variance followed by Neuman-Keuls multiple test.

RESULTS

Effect of Manganese on the ΔΨm in Cultured Astrocytes—To determine whether manganese treatment of cultured astrocytes was associated with a change in the ΔΨm, a consequence of the MPT, we treated astrocytes with various concentrations of manganese acetate (10–100 μM) for 3–24 h and determined the ΔΨm by TMRE fluorescence. Astrocytes exposed to 100 μM manganese showed a progressive dissipation of the ΔΨm as demonstrated by decreased TMRE fluorescence (Fig. 1). At 30 and 60 min, no change in TMRE fluorescence was observed, whereas at 90 min there was 20% (p < 0.05) reduction, which further declined to 50% (p < 0.01) at 3 h. This decline was maintained for up to 12 h. These changes in TMRE fluorescence were prevented by pretreatment with CsA (1 μM), consistent with induction of the MPT.

Treatment of astrocytes with lower concentrations of manganese (10–25 μM) also dissipated the ΔΨm, but the effect was delayed and of lesser magnitude. For example, at 25 μM manganese concentration, dissipation of the ΔΨm was not evident until 12 h, the magnitude of which was less compared with 100 μM (Fig. 2). Similarly, 10 μM manganese showed a significant
decrease in the ΔΨₘ only at 24 and 48 h (Fig. 2).

We further examined whether manganese was causing severe cell injury or death that might explain the collapse of the ΔΨₘ. Cells were exposed to manganese (25–100 μM) for 1 day, fixed in 10% neutral formalin for 30 min, stained with May-Grunwald/Giemsa and examined by light microscopy. Manganese at the concentrations and time employed above did not cause morphological alterations in cultured astrocytes (data not shown).

**Effect of Manganese on the ΔΨₘ in Neurons**—Cultured neurons, even after exposure to 100 μM manganese, showed relatively little decrease in the ΔΨₘ, and the time course of this reduction was delayed as compared with astrocytes. At 3 h, manganese had no effect on TMRE fluorescence, whereas at 6 h it was less decreased compared with astrocytes. CsA (1 μM) significantly blocked the manganese-induced decrease in TMRE fluorescence in both astrocytes and neurons.

**Effect of Manganese on the MPT in Astrocytes Using Calcein Fluorescence**—We also employed the calcein fluorescence method to demonstrate the induction of the MPT. The findings in mitochondrial calcein fluorescence were similar to that observed with TMRE. Astrocytes exposed to 100 μM manganese showed a progressive dissipation of calcein fluorescence. At 30 and 60 min, no change in fluorescence was detected, whereas at 90 min there was a 20% (p < 0.05) reduction, which further declined to 50% (p < 0.01) at 6 h and remained at that level for up to 12 h (Fig. 4). This decrease of calcein fluorescence was also prevented by CsA (1 μM). Studies employing a lower concentration (10 and 25 μM) of manganese also paralleled the TMRE findings (Fig. 5).

**Effect of Antioxidants on TMRE and Calcein Fluorescence in Astrocytes**—Astrocytes were pretreated with the antioxidant SOD (25 units/ml), the iron-chelating agent deferoxamine (DFX, 40 μM), the spin-trapping agent N-tert-butyl-α-phenyl-nitron (250 μM), and vitamin E (250 μM) and exposed to manganese (100 μM) for 6 h. DFX completely blocked the decrease in TMRE and calcein fluorescence in manganese-exposed astrocytes, whereas SOD and vitamin E partially blocked this effect (Figs. 5–9).

**DISCUSSION**

This study demonstrated that exposure of astrocyte cultures to pathophysiological concentrations of manganese acetate resulted in a significant dissipation of the ΔΨₘ and reduced the mitochondrial calcein fluorescence in a time- and concentration-dependent manner. These changes were completely blocked by
pretreatment with CsA, indicating the involvement of the MPT in this process. The concentrations (25–100 μM) of manganese employed in the present study correspond to levels found in autopsied brains from patients with cirrhosis, a condition associated with deposition of high levels of manganese in the basal ganglia (5, 6).

Neurons exposed to high concentrations of manganese (100 μM) showed only a slight induction of the MPT as compared with astrocytes. The reason for the selective vulnerability of astrocytes to manganese is not known. Aschner et al. (22) and Tholey et al. (23) have reported the presence of high affinity transporters for free manganese in astrocytes as well as the high capacity of astrocytes to accumulate manganese. In neurons, on the other hand, transferrin-bound manganese is preferentially transported rather than free manganese (24, 25).

Therefore, readily available free manganese in astrocytes may be one reason for the greater vulnerability of astrocytes over neurons to manganese toxicity. Alternatively, because most of the manganese resides in mitochondria (26), mitochondrial heterogeneity among neurons and astrocytes (27) may also explain this phenomenon. In this context, it is noteworthy that Fiskum (28) demonstrated a greater resistance of neuronal mitochondria to the effects of Ca²⁺ overload on the induction of oxidative stress and subsequent opening of the permeability transition pore, as compared with astrocytic mitochondria.

Consistent with the selective vulnerability of astrocytes to manganese toxicity, several reports indicate that morphological changes in astrocytes (Alzheimer type II astrocytosis) are the dominant neuropathological features of manganese neurotoxicity (29, 30). This astrocytic change is also the characteristic pathological finding in hepatic encephalopathy, a condition in which...
manganese has been implicated in its pathogenesis (5, 6). Although earlier work has not directly demonstrated the induction of the MPT by manganese either in intact cells or in isolated mitochondria, Allshire et al. (31) reported that manganese stimulates Ca\textsuperscript{2+} flux into isolated mitochondria, a process critical to the induction of the MPT. Studies by Bernardi et al. (32) demonstrated a competition between manganese and calcium for the same binding site on the permeability transition pore. Other studies (33) also indicated that manganese inhibits Ca\textsuperscript{2+} efflux from mitochondria, suggesting the possibility that mitochondria are undergoing the MPT following manganese exposure. More recently, Zhang et al. (34) have shown that high levels of manganese chloride (1 mM) caused a significant dissipation of the ΔΨ\textsubscript{m} in isolated rat brain mitochondria, consistent with the induction of the MPT.

Although mechanisms involved in the induction of the MPT by manganese are not known, manganese has been shown to induce oxidative stress. Manganese, being a transition element, is capable of existing in different oxidation states, including Mn\textsuperscript{2+}, Mn\textsuperscript{3+} and Mn\textsuperscript{4+}. The transitional shift of Mn\textsuperscript{2+} to Mn\textsuperscript{3+} could lead to increased oxidant capacity of this metal. Accordingly, recent reports indicate that Mn\textsuperscript{3+} was more toxic compared with Mn\textsuperscript{2+} (35). Treatment of astrocytes with manganese increased free radical production and decreased the activities of key antioxidant enzymes, SOD and glutathione peroxidase (11). More recently, Zhang et al. (34) reported the increased production of free radicals in isolated rat brain mitochondria exposed to manganese, in addition to the inhibition of various electron transport chain enzymes.

Our data demonstrated that pretreatment of astrocyte cultures with antioxidants either completely blocked or significantly attenuated the manganese-induced MPT. Pretreatment with DFX, an iron-chelating agent, completely blocked the MPT by manganese. Consistent with this observation, HaMaI et al. (36) demonstrated that DFX was able to block manganese-induced free radical production in synaptoneurotic and nonsynaptoneurotic mitochondria. They suggested that DFX, by binding to Mn\textsuperscript{3+}, could sequester manganese and thereby prevent free radical formation. Conversely, Roth et al. (37) reported that DFX potentiated manganese-induced cell death in PC12 cells by inhibiting energy metabolism. Such discrepancy may be because Roth et al. (37) employed a much higher concentration (300 μM) of MnCl\textsubscript{2} for a longer period of time (24–48 h), whereas our studies employed only 100 μM manganese acetate for up to 6 h. Moreover, the DFX concentration (10 μM) employed by Roth et al. (37) was much lower than that used in our study (40 μM). In the present study, complete blocking of the manganese-induced MPT by DFX, in conjunction with significant attenuation of the same by SOD and vitamin E (Fig. 9), indicates a major role of oxidative stress in the induction of the MPT by manganese. These findings are consistent with reports demonstrating the attenuation of the MPT by various antioxidants in the setting of oxidative stress (38, 39). Taken together, these findings clearly suggest that oxidative stress plays a major role in the induction of the MPT by manganese.

Another potential mechanism by which manganese can induce the MPT is by inhibition of Ca\textsuperscript{2+} efflux from the mitochondria. Manganese is transported into mitochondria by the Ca\textsuperscript{2+} uniporter (40). Other studies have shown that excessive accumulation of manganese in mitochondria results in inhibition of both Na\textsuperscript{+}-dependent and -independent Ca\textsuperscript{2+} efflux (33). Such mitochondrial Ca\textsuperscript{2+} overload may result in induction of the MPT (14, 41).

Despite induction of the MPT in cultured astrocytes and to a lesser extent in cultured neurons by manganese acetate, there were no significant morphological changes or evidence of cell death observed at the concentrations (25–100 μM) of manganese acetate employed in this study. These data are consistent with our earlier observations (42) that used manganese chloride (100 μM) as well as with those of other investigators (11, 43). Although it is generally believed that induction of the MPT leads to cell death (apoptosis and or necrosis), several reports indicate that the MPT is a reversible process (16, 44). Studies by Petronilli et al. (45) demonstrated complete reversibility of the MPT in isolated mitochondria over several minutes. Employing intact osteosarcoma cells, Minamikawa et al. (46) show a complete reversibility of the MPT without any evidence of cell death. Similarly, it has been reported that astrocytes subjected to oxygen-glucose deprivation showed induction of the MPT without evidence of cell death (47). In this regard, it is noteworthy that our preliminary studies indicate that induction of the MPT in astrocytes when exposed to 100 μM manganese acetate for 6 h was completely reversed 48 h after removal of the manganese in the culture medium.

Because the opening of the permeability transition pore is an “all or none” phenomenon (14), it is the number of mitochondria in a cell that are susceptible to pore opening rather than the degree of pore opening that is considered critical in the mechanism of cell death (apoptosis/necrosis) associated with the MPT (48). The number of mitochondria undergoing the MPT will in turn depend on the degree of mitochondrial insult caused by an inducing agent (e.g. manganese). Therefore, it is conceivable that higher concentrations of manganese treatment for longer time periods result in cell death, possibly mediated by the MPT.

In summary, we have shown that manganese preferentially induces the MPT in cultured astrocytes. Cultured neurons were far less affected by manganese exposure compared with astrocytes. The manganese-induced MPT was blocked by antioxidants, suggesting the potential involvement of oxidative stress in this process. Induction of the MPT by manganese and associated mitochondrial dysfunction in astrocytes may represent key mechanisms in manganese neurotoxicity.

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