Manganese Induces Oxidative Impairment in Cultured Rat Astrocytes

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Received February 21, 2007; accepted April 18, 2007

Excessive free radical formation has been implicated as a causative factor in neurotoxic damage associated with exposures to a variety of metals, including manganese (Mn). It is well established that Mn accumulates in astrocytes, affecting their ability to indirectly induce and/or exacerbate neuronal dysfunction. The present study examined the effects of Mn treatment on the following endpoints in primary astrocyte cultures: (1) oxidative injury, (2) alterations in high-energy phosphate (adenosine 5′-triphosphate, ATP) levels, (3) mitochondrial inner membrane potential, and (4) glutamine uptake and the expression of glutamine transporters. We quantified astrocyte cerebral oxidative damage by measuring F2-isoprostanes (F2-IsoPs) using stable isotope dilution methods followed by gas chromatography–mass spectrometry with selective ion monitoring. Our data showed a significant \( p < 0.01 \) elevation in F2-IsoPs levels at 2 h following exposure to Mn (100 \( \mu \)M, 500 \( \mu \)M, or 1mM). Consistent with this observation, Mn induced a concentration-dependent reduction in ATP and the inner mitochondrial membrane potential (\( \Delta \Psi_{m} \)), measured by the high pressure liquid chromatography method and the potentiometric dye, tetramethyl rhodamine ethyl ester, respectively. Moreover, 30 min of pretreatment with Mn (100 \( \mu \)M, 500 \( \mu \)M, or 1mM) inhibited the net uptake of glutamine (GLN) (\(^{3}H\)-glutamine) measured at 1 and 5 min. Expression of the messenger RNA coding the GLN transporters, SNAT3/SN1 and SNAT1, was inhibited after 100 and 500 \( \mu \)M Mn treatment for 24 h. Our results demonstrate that induction of oxidative stress, associated mitochondrial dysfunction, and alterations in GLN/glutamate cycling in astrocytes represent key mechanisms by which Mn exerts its neurotoxicity.

Key Words: astrocyte; manganese; F2-isoprostanes; mitochondria; ATP; \( \Delta \Psi_{m} \); glutamine.

Manganese (Mn) is a naturally occurring trace metal commonly found in the environment. It is essential for maintaining the proper function and regulation of many biochemical and cellular reactions (Takeda, 2003). Despite its essentiality, Mn is a common environmental contaminant, which can exert neurotoxic activity in human cells. Major sources of environmental contamination with Mn include the manufacturing of alloys, steel, iron, and fertilizers, as well as mining operations (Aschner, 2000). Excessive accumulation of Mn in specific brain areas, the substantia nigra, the globus pallidus (GP), and the striatum, produce neurotoxicity resulting in a degenerative brain disorder, referred to as manganism. It is characterized by clinical signs and morphological lesions similar to those seen in Parkinson’s disease (PD) (Racette et al., 2001). In addition to targeting similar brain areas, PD and manganism share multiple common mechanisms leading to dopaminergic neurodegeneration, namely, mitochondrial dysfunction, aberrant signal transduction, oxidative stress, protein aggregation, and the activation of cell death pathways (Dobson et al., 2004; HaMai and Bondy, 2004; Kitazawa et al., 2005; Latchoumycandane et al., 2005).

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). 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 F2-isoprostanes (F2-IsoPs), prostaglandin-like molecules produced by free radical–mediated peroxidation of arachidonic acid (Morrow and Roberts, 1999). These biomarkers of oxidative stress have been investigated in several in vivo models associated with free radical damage, but not within the context of Mn-induced neurotoxicity. In addition, ROS generation, the ensuing decrease in mitochondrial membrane potential, and the depletion of high-energy phosphates affect mitochondrial permeability transition (MPT), leading to organelle swelling, disruption of the outer membrane, and release of numerous apoptogenic factors into the cytosol (Green and Reed, 1998).

Given the propensity of astrocytes to accumulate Mn, it is reasonable to postulate that they represent an initial site of Mn-induced damage. Astrocytes perform functions essential for normal neuronal activity, including glutamate (GLU) uptake (80% of synaptic GLU), glutamine (GLN) release, \( K^{+} \) and \( H^{+} \)...
buffering and water transport (Kimelberg and Aschner, 1998). A principal neuromodulatory role of astrocytes is the control over GLU–GLN homeostasis. GLU is metabolized to GLN by the astrocyte-specific enzyme, glutamine synthase (GS). This GLU–GLN pathway constitutes the source of a GLU pool in the brain. GS contains eight Mn ions per octamer and accounts for approximately 80% of the total endogenous Mn in the brain. Unlike neurons, astrocytes have the ability to concentrate Mn to levels 50-fold higher than the culture media (Aschner et al., 1992); this provides a mechanism by which Mn concentrations in the astrocytic cytosol attain the range required for optimal activation of GS, thereby ensuring optimal production of GLN for subsequent neuronal uptake and metabolism into γ-amino-butyric acid (GABA). However, excessive Mn uptake and subsequent sequestration by mitochondria may result in cellular dysfunction as discussed above.

GLN efflux from astrocytes is mediated by the sodium-coupled neutral amino acid transporter 3 (SNAT3, SN1), a System N amino acid transporter localized to perisynaptic astrocytes, which specifically transports only GLN, histidine, and asparagine (Chaudry et al., 2002). System N is a Na\(^+\)-glutamine symporter and an H\(^+\) antiporter. Another GLN-accepting amino acid transporter more abundantly expressed in astrocytes than in neurons is ASCT2 (Dolinska et al., 2004). System ASC is a sodium-dependent antiporter which exists as two different isoforms termed ASCT1 and ASCT2, with ASCT2 having an affinity for GLN. GLN uptake in neurons is also mediated by sodium-dependent transporters of the System A family, two of which, SNAT1 (GlnT, SAT1, ATA1, SA2) and SNAT2 (SAT2, TAT2), are known to modulate GLU and/or GABA recycling, and, thereby, synaptic function (Heckel et al., 2003). A previous study from our laboratory has shown that exposure of astrocytes to acrylamide or methylmercury affects astrocytic GLN uptake and expression of messenger RNA (mRNA) encoding for GLN transporters (Aschner et al., 2005; Wu et al., 2005; Yin et al., 2007). Furthermore, excessive accumulation of GLN and alterations in GLN recycling, as a result of interference with mitochondrial function, are deleterious to brain function (Albrecht et al., 2007).

In view of the essential function of astrocytes for maintaining control over the composition of the extracellular fluid and normal neuronal activity, we evaluated Mn’s effects on oxidative stress; alterations in adenosine 5’-triphosphate (ATP) levels; mitochondrial inner membrane potential (ΔΨ\(_{m}\)); GLN uptake; and expression of SNAT3, ASCT2, and SNAT1 mRNA.

**MATERIALS AND METHODS**

**Materials.** L-[G-\(^{3}H\)]-GLN (specific activity: 49.0 Ci/mm mol) was purchased from Amersham Biosciences (Piscataway, NJ). Manganese chloride (MnCl\(_2\)) and ATP standards were purchased from Sigma Chemical Co. (St Louis, MO). Minimal essential medium (MEM) with Earle’s salts, heat-inactivated horse serum, penicillin, streptomycin, and tetramethyl rodamine ethyl ester (TMRE) were purchased from Invitrogen (Carlsbad, CA). \(^{4}H\)-labeled 15-F\(_{2}\)-IsoP internal standard and prostaglandin F\(_{2}\) methyl ester were purchased from Cayman chemicals (Ann Arbor, MI).

**Primary astrocytes culture.** The procedure for isolation and culturing of astrocytes from cerebral cortices of newborn (1 day old) Sprague–Dawley rats (Aschner et al., 1994) was approved by the institutional committee for animal research and was performed in accordance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals. Briefly, rat pups were decapitated, and the cerebral cortices were removed. After removal of the meninges, the cerebral cortices were digested with bacterial neutral protease (dispace, Invitrogen), and astrocytes were recovered by repeated removal of dissociated cells from brain tissues. Twenty-four hours after the initial plating in 6- and 12-well plates, the media were changed to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air/5% CO\(_2\) incubator for 3–4 weeks in MEM with Earle’s salts supplemented with 10% heat-inactivated horse serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The media were changed twice per week. The surface-adhering monolayer cultures were > 95% positive for the astrocytic marker, glial fibrillary acidic protein.

**F\(_{2}\)-IsoPs assay.** Total F\(_{2}\)-IsoPs were measured in primary astrocyte cultures exposed to 100μM, 500μM, or 1mM of MnCl\(_2\) for 30 min, 2 or 6 h by using gas chromatography/mass spectrometry with selective ion monitoring (Morrow and Roberts, 1999). Briefly, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated, and then subjecteed to chemical saponification using 15% KOH to hydrolyze bound F\(_{2}\)-IsoPs. The cell lysates were adjusted to a pH of 3, followed by the addition of 0.1 ng of \(^{3}H\)-labeled 15-F\(_{2}\)-IsoP internal standard. F\(_{2}\)-IsoPs were subsequently purified by C\(_{18}\) and silica Sep-Pak extraction and by thin layer chromatography. They were then analyzed by pentfluorobenzenyl ester, a trimethylsilyl ether derivative, via gas chromatography, negative ion chemical ionization mass spectrometry.

Mn concentrations in this study (100μM, 500μg/ml, or 1mM) were determined based on the relevant toxic Mn effect on mammalian cells as described in the literature. For example, weekly injections of Mn over a 3-month period (0, 2.25, 4.5, or 9 g) in the striatum and the GP of monkeys have been shown to produce dose-related clinical signs, which are more severe in the higher dose ranges (Suzuki et al., 1975). At the highest dose, the Mn concentration is increased 12-fold in the striatum and ninefold in the GP. The “physiological range” (no symptoms) is 75–100μM, and clinical signs increase in frequency and severity above this level, suggesting that this concentration is near the threshold of toxicity.

**Measurement of ATP.** Levels of ATP were analyzed by an isocratic reversed-phase high pressure liquid chromatography (HPLC) method (Yang et al., 2002). ATP was extracted from control and Mn-exposed astrocytes by adding 950 μl of ice-cold perchloric acid (0.2M) containing Na-ehtylenediamine-minitetraacetic acid (1mM) to the primary astrocyte culture plates immediately after the medium was removed. The cells were then scraped off the bottom of the plates, and the acid extract was transferred to a microcentrifuge tube. The acid extract was neutralized with 170 μl of KOH; 2M and centrifuged at 9000 × g for 5 min to remove fine precipitates of perchlorate (KClO\(_4\)). The supernatants were stored at −20°C before being subjected to ATP determination. The concentration of ATP was determined in a 15 μl of sample extract injected into HPLC with ultraviolet detector and 0.1M of ammonium dihydrogen phosphate (pH 6.0) containing 1% ethanol as a mobile phase. Using the Symmetry Shield C-18 column and a flow rate of 0.6 ml/min, the peak of ATP production was eluted at a retention time of 3.462 min and recorded at 206 nm.

**Measurement of mitochondrial membrane potential (ΔΨ\(_{m}\)).** The ΔΨ\(_{m}\) was measured with the fluorescent dye, TMRE (Rao and Norenberg, 2004). At the end of the treatments with 100μM, 500μM, or 1mM of Mn for 1 or 6 h, the culture medium was removed (duplicate plates per experiment; repeated three times using different astrocyte isolations), and the cells were loaded with TMRE at a final concentration of 50nM in N-2-hydroxy-ethylpiperazine N’-2-ethansulfonic acid (HEPES) buffer for 20 min at 37°C in a 5% CO\(_2\) incubator.
Cells were rinsed with phosphate-buffered saline and examined with a Zeiss inverted fluorescent microscope (Zeiss Axiosvert S100, Carl Zeiss Micro-Imaging Inc., Thornwood, NY) equipped with a cooled digital camera (Photometrics CoolSNAP, Roper Scientific Photometrics, Tucson, AZ). Images of various fields in each plate were captured at ×10 magnification with the digital camera. Fluorescent intensities were calculated in eight randomly selected fields per experiment and were 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), and the mean pixel value in each image field was expressed as mean ± SEM. The fluorescent intensities were expressed as percent fluorescence change from control; each experiment was performed in duplicate plates and repeated three times in astrocytes derived from independent isolations.

**Analysis of 3H-glutamine uptake in astrocytes.** 3H-glutamine uptake was assayed as previously described (Allen et al., 2001). Astrocytes were studied at 3 weeks when the monolayers were confluent. Cells in six-well plates were washed three times with 2 ml of fresh sodium-HEPES buffer consisting of the following: 122 mM of NaCl, 3.3 mM of KCl, 0.4 mM of MgSO4, 1.3 mM of CaCl2, 1.2 mM of KH2PO4, 10 mM of glucose, and 25 mM of HEPES adjusted to a pH of 7.4 with 10 mM of NaOH. In preincubation experiments, cells were pre-treated in Na-HEPES buffer only, or with Na-HEPES buffer containing Mn (100 μM, 500 μM, or 1 mM) for 30 min in a 37°C, 95% air/5% CO2 incubator. Cells were then washed three times with 2 ml of Na-HEPES buffer. Thereafter, 1 ml of prewarmed buffer containing 1 μCi/ml of L-[G-3H]-GLN in the presence of unlabeled GLN (at a final concentration of 50 μM) was added to each well, and GLN uptake was measured at 1 and 5 min at room temperature. At each time point, the reactions were terminated by aspirating the buffer from the well, followed by five washes with 2 ml of ice-cold mannitol buffer (290 mM of mannitol, 10 mM of Tris-nitrate, 0.5 mM of Ca(NO3)2; pH adjusted to 7.4 with KOH). At the end of the experiment, cells were lysed in 2 ml of 1 M NaOH. An aliquot of 25 μl was used for protein determination by the bicinchoninic acid protein assay (Pierce, Rockford, IL). An aliquot of 750 μl was used for radioactivity measurement. Uptake of GLN was expressed as cpm/mg of protein.

**Reverse transcription-polymerase chain reaction.** Total RNA from cell cultures exposed to Mn concentration of 100 μM, 500 μM, or 1 mM for 24 h was extracted using Trizol (Invitrogen). RNA (2 μg) was transcribed using Superscript II (Invitrogen). The primers were obtained from the Laboratory of DNA Sequencing, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. Each complementary DNA (2 μl) was amplified by PCR using the primers for rat amino acid transporters: SNAT3, SNAT1, and ASCT2. The expression was quantitatively related to the gene coding for the constitutive protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequence of primers and the lengths of the products are outlined in Table 1. After 30 cycles of amplification (1 min at 94°C, 1 min at 59°C for SNAT3, and 55°C for ASCT2 and SNAT1 and 1 min at 72°C using Biometra Thermocycler), the PCR products were recorded using the NucleoVision system (Nucleotech, San Mateo, CA), and densitometric analysis was carried out using the GeMExpert 4.0 program.

**Statistical analysis.** Measurements of F2-IsoPs, ATP, and mitochondrial membrane potential, as well as experiments with 3H-glutamine uptake were conducted in duplicate or triplicate wells/experiment, and the mean from three to four independent experiments was used for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test with statistical significance set at *p* < 0.05. All analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA).

### RESULTS

**Effects of Mn on F2-IsoPs Formation in Cultured Astrocytes**

We tested the ability of Mn to induce oxidative stress in primary astrocyte cultures by measuring levels of F2-IsoPs, a lipid peroxidation biomarker of oxidative injury. Neonatal rat primary astrocytes exposed to 500 μM or 1 mM of MnCl2 for 30 min, 2 or 6 h showed significant increases in F2-IsoPs levels compared to controls (Fig. 1). Astrocyte cultures exposed to 1 mM of Mn for 30 min showed a fivefold increase in F2-IsoPs compared to the control level (137.3 ± 4.3 pg/mg protein). The lowest MnCl2 concentration (100 μM) induced a significant increase in markers of oxidative stress at 2 h, but not at 30 min or 6 h following Mn exposure.

**Characterization of Cellular ATP Following Mn Exposure**

Total cellular ATP levels were measured in MnCl2-exposed astrocyte cultures as an additional indicator of overt cellular toxicity and mitochondrial dysfunction. ATP levels were significantly decreased in astrocytes exposed for 30 min to the full range of investigated Mn concentrations (Fig. 2). The greatest depletion of ATP (63%) was seen in astrocytes exposed for 30 min to 1 mM of MnCl2. Astrocyte cultures exposed for

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**TABLE 1**

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**FIG. 1.** Effects of MnCl2 on F2-IsoPs formation in cultured astrocytes. Rat primary astrocyte cultures were incubated at 37°C in the absence or presence of MnCl2 (100 μM, 500 μM, or 1 mM), and F2-IsoPs levels were quantified at 30 min, 2 h, and 6 h. Data represent the mean ± SEM from three independent experiments. *p* < 0.05 versus control by one-way ANOVA followed by Bonferroni multiple comparison tests.
30 min or 2 h to 500 μM of Mn showed similar levels of ATP depletion to those exposed for 30 min or 2 h to only 1 mM of Mn. In contrast, astrocytes exposed for 2 h to the lowest concentrations of Mn (100 μM) did not show significant reductions in ATP levels.

**Effects of Mn on the ΔΨ<sub>m</sub> in Cultured Astrocytes**

The ΔΨ<sub>m</sub>, an indicator of mitochondrial dysfunction, was evaluated in astrocytes exposed to Mn. Treatment of astrocytes with 100 μM, 500 μM, or 1 mM of Mn for 1 or 6 h resulted in significant dissipation of the ΔΨ<sub>m</sub>, as demonstrated by decreased mitochondrial TMRE fluorescence (Figs. 3A and 3C). Quantification of TMRE fluorescence intensities revealed that all the concentrations of Mn investigated induced significant dissipation of the ΔΨ<sub>m</sub> following 1 or 6 h of exposure (Figs. 3B and 3D). Fluorescence intensities of astrocytes exposed to 100 μM, 500 μM, or 1 mM of Mn for 1 h were indistinguishable from intensities of astrocytes exposed to the same Mn concentrations for 6 h.

**GLN Uptake Inhibition by Mn in Cultured Astrocytes**

Compared with control uptake rates (100%), pretreatment of astrocytes for 30 min with Mn inhibited the net uptake of GLN in a concentration-dependent manner. All Mn treatments significantly inhibited GLN astrocytic uptake at 1 min and 5 min (Fig. 4). Furthermore, 1 mM of Mn treatment induced greater inhibition of GLN uptake at 1 min compared to the inhibition induced at 5 min.

**Influence of Mn on Expression of SNAT3, ASCT2, and SNAT1 mRNA**

To explore molecular mechanisms associated with the effects of Mn on GLN uptake, we measured astrocytic mRNA expression of amino acid transporters by reverse transcription-PCR (RT-PCR). Figure 5 depicts the effects of Mn treatment on the mRNA expression of SNAT3, ASCT2, and SNAT1. The bars display the ratios of SNAT3, ASCT2, and SNAT1 to GAPDH, a constitutively expressed marker. Treatments with 100 and 500 μM of Mn for 24 h significantly reduced the mRNA expression of SNAT3 and SNAT1, but did not reduce the mRNA expression of ASCT2 (Fig. 5).

**DISCUSSION**

The present study provides a direct link between mitochondrial dysfunction and oxidative stress due to Mn neurotoxicity. To our knowledge, this is the first study to investigate Mn-induced neurotoxicity in astrocytes by quantifying biomarkers of oxidative damage (F2-IsoPs) and energy metabolites (ATP). Our results show that increased lipid peroxidation, depletion of high-energy phosphates, and the collapse of the mitochondrial inner membrane potential (ΔΨ<sub>m</sub>) represent early events and key mechanisms in Mn-induced astrocytic dysfunction.

We investigated Mn-induced oxidative stress in astrocytes by measuring F2-IsoPs (Morrow and Roberts, 1999), a group of arachidonic acid-derived prostanoid isomers generated by free radical damage due to arachidonic acid that are not extensively metabolized in situ. Moreover, the National Institute of Environmental Health Science Biomarkers Oxidative Stress Study (Kadiiska et al., 2005) recently proposed that F2-IsoPs are the most reliable biomarkers of oxidative damage. Astrocytes exposed to Mn concentrations known to elicit neurotoxic effects (500 μM or 1 mM), showed significant elevations in F2-IsoPs levels at all investigated exposure times (Fig. 1). Thus, increases in ROS, which are generated by electron leak from the electron transport chain (ETC) (Turrens and Boveris, 1980), potentially damaging mitochondria directly or through the effects of secondary oxidants like superoxide, H₂O₂, or peroxynitrite (ONOO⁻), mediate Mn-induced oxidative damage. Moreover, superoxide produced in the mitochondrial ETC may catalyze the transition shift of Mn<sup>2+</sup> to Mn<sup>3+</sup> through a set of reactions similar to those mediated by superoxide dismutase and thus lead to the increased oxidant capacity of this metal (Gunter et al., 2006). Consequent oxidative damage produces an array of deleterious effects: it may cause structural and functional derangement of the phospholipids bilayer of membranes, disrupt energy metabolism, metabolite biosynthesis, calcium and iron homeostasis, and initiate apoptosis (Attardi and Schatz, 1988; Uchida, 2003; Yang et al., 1997).

Interestingly, following exposure to a low MnCl₂ concentration (100 μM), levels of F₂-IsoPs were significantly increased only at 2 h but were indistinguishable from controls (137.3 ± 4.3 pg/mg protein) at 30 min and 6 h (Fig. 1). A similar response of F₂-IsoPs increase followed by full recovery was observed in our in vivo models of innate immunity- and excitotoxicity-mediated cerebral oxidative damage (Milatovic...
et al., 2003, 2005). F2-IsoPs were not significantly increased in the cerebra of mice following the intracerebroventricular (icv) injection of lipopolysaccharide (LPS, 5 μg in 5 μl of saline) at 10 h but were significantly increased at 24-h postinjection (Milatovic et al., 2003). By 72-h postinjection, F2-IsoPs values in LPS-treated mice had returned to baseline. Thus, oxidative stress has been implicated not only in metal-induced neurotoxicity (Dobson et al., 2004), but also in models of inflammation and excitotoxicity, as well as in a wide range of neurodegenerative conditions, such as PD and Alzheimer’s disease (Albers and Beal, 2000). A previous report showed that neurons exposed to fuel additive, methylcyclopentadienyl manganese tricarbonyl, or MnCl2 are susceptible to oxidative stress, energy failure associated with mitochondrial dysfunction (Zwingmann et al., 2003), and mitochondria-induced apoptosis (Anantharam et al., 2002). Thus, it is likely that the oxidative impairment of astrocytic functions may indirectly induce and/or exacerbate neuronal dysfunction.

Consistent, and preceding the Mn-induced increased on F2-IsoPs levels (Fig. 1), we noted an early decrease in astrocytic ATP levels. Higher concentrations of MnCl2 (500μM and 1mM) induced significant (p < 0.01) decreases in ATP levels at all investigated exposure times (Fig. 2). As a consequence, ATP depletion or a perturbation in energy metabolism might diminish the ATP-requiring neuroprotective action of astrocytes, such as GLU and GLN uptake and free radical scavenging (Rao et al., 2001). In addition, depletion of high-energy phosphates may affect intracellular Ca2+ in astrocytes through mechanisms involving the disruption of mitochondrial Ca2+ signaling. This assertion is supported by data that clearly

**FIG. 3.** Quantitation of TMRE fluorescent intensities. Cultured astrocytes were exposed to MnCl2 at various concentrations (0, 100μM, 500μM, or 1mM) for 1 h (A and B) and 6 h (C and D), and the fluorescent images were quantified as described in Section 2. Values are expressed as mean ± SEM of 24 random fields in each group. *p < 0.05, ***p < 0.001 versus control; (A) image after 1 h of exposure; (C) image after 6 h of exposure.
show that Mn inhibits Na\(^+\)-dependent Ca\(^{2+}\) efflux (Gavin et al., 1990) and respiration in brain mitochondria (Zhang et al., 2004), both critical to maintaining normal ATP levels and ensuring adequate intermitochondrial signaling.

We further evaluated Mn-induced cytotoxicity in astrocytes by measuring lactate dehydrogenase (LDH) release from injured cells into the culture medium, as well as mitochondrial function, using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The studies revealed that Mn exposure induced time- and concentration-dependent increase of LDH in astrocytes exposed to 1mM Mn for 2 h showed more than twofold increase in LDH and 30% decrease in MTT compared to controls.

Another consequence of increased oxidative stress and mitochondrial energy failure is the induction of the MPT, a Ca\(^{2+}\)-dependent process characterized by the opening of the permeability transition pore in the inner mitochondrial membrane. This process results in increased permeability to protons, ions, and other solutes (Zoratti and Szabo, 1995), which, consequently, leads to a collapse of the mitochondrial inner membrane potential (\(\Delta \Psi_m\)). Loss of the \(\Delta \Psi_m\) results in colloid osmotic swelling of the mitochondria matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and further generation of ROS. Our experiments demonstrated a concentration-dependent effect of Mn on the mitochondrial inner membrane potential in cultured astrocytes (Fig. 3). This effect was noted already at 1-h postexposure to Mn, consistent with the reduction in ATP (Fig. 2) and increased production of F2-IsoPs (Fig. 1). Additional experiments in which we evaluated fluorescence intensities of astrocytes exposed to 500\(\mu\)M of Mn for 30 min demonstrated that dissipation of the \(\Delta \Psi_m\) was indistinguishable between 30 min and 1 h exposure. Studies by Zhang et al. (2004) have shown that high levels of MnCl\(_2\) (1mM) cause a significant dissipation of the \(\Delta \Psi_m\) in isolated rat brain mitochondria, consistent with induction of the MPT. Our results are also in agreement with an earlier study by Rao and Norenberg (2004), which found that Mn causes a dissipation of the \(\Delta \Psi_m\) in a concentration- and time-dependent manner in astrocytes. The mechanism(s) by which Mn induces the MPT in astrocytes is not completely understood; however, increased production of ROS and associated oxidative stress are generally considered major factors of MPT induction (Castilho et al., 1995; Rao and Norenberg, 2004).

Our results show that pretreatment of astrocytes for 30 min with Mn inhibits the initial net uptake of GLN at 1 and 5 min in a concentration-dependent manner (Fig. 4). These observations are consistent with our findings that Mn decreases intracellular ATP as early as 30 min after exposure (Fig. 2). While one could certainly extend the GLN uptake studies to hours, we believe that the initial rates of uptake can be masked under those conditions, because it is hard to differentiate between frank uptake effects of Mn versus those on GLN efflux. As several of the GLN transporters are sodium-dependent (systems A, ASC, and N), dissipation of ionic gradients across membranes and the intracellular accumulation of sodium are consistent with reduced uptake of GLN in Mn-treated astrocytes. GLN is an important amino acid that plays a pivotal role in neuron–glia interactions, particularly in the turnover of the transmitter pool of GLU and GABA, the principal CNS excitatory and inhibitory neurotransmitters (Conti and Weinberg, 1999). Since neurons are not capable of anaplerosis, it is essential that they obtain precursors from astrocytes for transmitter synthesis. Glutamatergic and GABAergic neurons use glial GLN as precursors for neurotransmitter synthesis as a part of the GLU–GLN cycle (Sonnenthal et al., 1997). After uptake by astrocytes, GLU can either be converted to glutamine directly by glutamine synthetase, or can enter the tricarboxylic acid cycle after conversion to 2-oxoglutarate for energy production and/or the synthesis of other metabolites. Inhibition of GLN uptake in Mn-treated astrocytes is, therefore, likely to reflect...
the decreased ability of cells for maintaining neuronal GLN and GABA pools as well as the generation of releasable GLN and GABA (Albrecht et al., 2007). In addition, our data also demonstrate that the inhibition of GLN uptake in Mn-treated astrocytes is accompanied by the inhibition of the expression of mRNA coding for SNAT3 and SNAT1 (Fig. 5). The GLN transport proteins, SNAT3 and SNAT1, are capable of mediating outward and inward transport of GLN, with the direction depending on the GLN and/or pH gradients (Chaudry et al., 2002). While cellular ATP levels are critical to Mn-induced GLN uptake kinetic, our results implicate impaired GLN shuttling between astrocytes and neurons as one of the possible causes of the derangement of amino acidergic neurotransmission known to be associated with Mn toxicity.

In summary, our studies demonstrate that the mechanisms of Mn-induced neurotoxicity are associated with the induction of oxidative stress, mitochondrial dysfunction, and alterations in GLU–GLN cycling. Once inside the astrocytes, Mn induced the effects of transient increases in ROS generation, simultaneous collapse of the mitochondrial membrane potential, and exacerbates the effects of existing defects in electron transport to slow ATP production. Releases of ROS mediate lipid peroxidation, which, consequently, diminish membrane permeability and affect GLN shuttling. Finally, we propose that oxidative stress generated through mitochondrial perturbation plays a key role in Mn-induced astrocytic dysfunction.

ACKNOWLEDGMENTS

This study was supported by Public Health Service Grants ES10563 (M.A.) and ES013730 (J.L.A.) from the NIH and Department of Defense W81XWH-05-1-0239 (M.A.).

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