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Manganese-Induced Cytotoxicity in Dopamine-Producing Cells☆

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Abstract

Manganese (Mn) is an essential metal that, at excessive levels in the brain, produces extrapyramidal symptoms similar to those in patients with Parkinson's disease (PD). In the present study, Mn toxicity was characterized in a human neuroblastoma (SK-N-SH) cell line and in a mouse catecholaminergic (CATH.a) cell line. Mn was demonstrated to be more toxic in the catecholamine-producing CATH.a cells ($EC_{50} = 60$ μM) than in non-catecholaminergic SK-N-SH cells ($EC_{50} = 200$ μM). To test the hypothesis that the sensitivity of CATH.a cells to Mn is associated with their dopamine (DA) content, DA concentrations were suppressed in these cells by pretreatment with α-methyl-para-tyrosine (AMPT). Treatment for 24 h with 100 μM AMPT decreased intracellular DA, but offered no significant protection from Mn exposure ($EC_{50} = 60$ μM). Additional studies were carried out to assess if Mn toxicity was dependent on glutathione (GSH) levels. CATH.a cells were significantly protected by the addition of 5 mM GSH (Mn $EC_{50} = 200$ μM) and 10 mM N-acetyl cysteine (NAC) (Mn $EC_{50} = 300$ μM), therefore, indirectly identifying intracellular ROS formation as a mechanism for Mn neurotoxicity. Finally, apoptotic markers of Mn-induced cell death were investigated. DNA fragmentation, caspase-3 activation, and apoptosis-related gene expression were studied in CATH.a cells. No internucleosomal fragmentation or caspase activation was evident, even in the presence of supraphysiological Mn concentrations. cDNA hydridization array analysis with two differing Mn concentrations and time points, identified no noteworthy mRNA inductions of genes associated with programmed cell death. In conclusion, DA content was not responsible for the enhanced sensitivity of CATH.a cells to Mn toxicity, but oxidative stress was implicated as a probable mechanism of cytotoxicity.

Keywords: CATH.a; Dopamine; DNA fragmentation; Manganese; Cytotoxicity

INTRODUCTION

Manganese (Mn) is an essential metal in both humans and animals. It serves as the functional moiety for many enzymes, such as pyruvate carboxylase (PC), glutamine synthetase (GS), and numerous peroxidases (Wedler, 1993). Although Mn deficiencies are rare, they can lead to seizures, impaired growth, skeletal abnormalities and impaired reproductive function (Critchfield et al., 1993; Wedler, 1993). Conversely, excessive exposure to Mn produces neurotoxicity (McMillan, 1999). The recent approval of methylcyclopentadienyl manganese tricarbonyl (MMT) as a fuel antiknock additive has raised concern regarding the potential for increased atmospheric concentrations of Mn, and by inference, increased Mn body burden in the population at large.

Symptoms of acute Mn toxicity are similar to those seen in patients with Parkinson's disease (PD); namely, gait imbalance, rigidity, tremors, bradykinesia, and

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dystonia (Barbeau et al., 1976). Unlike Parkinsonism, manganism also produces dystonia, a neurological sign associated with damage to the globus pallidus (Calne et al., 1994; Pal et al., 1999). Recognized differences between Parkinson's disease and manganism were also noted, particularly in manganism: (a) A less frequent resting tremor. (b) More frequent dystonia. (c) A particular propensity to fall backwards. (d) Failure to achieve a sustained therapeutic response to levodopa. (e) Failure to detect a reduction in fluorodopa uptake by positron emission tomography (PET; for further details see Calne et al., 1994). Given that Mn toxicity (manganism) shares many commonalities with PD, a dopaminergic cell disorder, and the fact that Mn accumulates in dopaminergic areas, it has been suggested that Mn neurotoxicity involves an imbalance of dopaminergic neurotransmission (Graham, 1978; Hornykiewicz, 1972; Neff et al., 1969; Newland et al., 1987). This imbalance could be the result of dopamine-mediated cell death, which prompts investigation into cellular mechanisms.

At the cellular level, it has been shown that Mn decreases antioxidants such as, thiols, catalase, and glutathione (GSH) (Archibald and Tyree, 1987; Donaldson, 1987; Liccione and Maines, 1988). This decrease in intracellular antioxidants is compounded by the observation by Sloot et al. (1996) that Mn induces ROS formation. Mn-induced elevated ROS formation and decreased antioxidant levels could tip the cellular balance in favor of toxicity. Antioxidants such as, ascorbic acid (AA), glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) and *N*-acetyl cysteine (NAC) decrease ROS, protecting cells from cellular damage and/or death. The antioxidant GSH is a low molecular mass thiol found in plant and animal cells that plays a role in protection against oxidative stress and reactive electrophiles (Meister, 1994; Meister and Anderson, 1984; Griffith and Mulcahy, 1999). GSH cannot cross cellular membranes, but intracellular pools can be increased when NAC, which does readily cross membranes, is deacetylated into cysteine, a precursor for GSH.

One potential mechanism for Mn neurotoxicity is through a form of programmed cell death, apoptosis. Apoptosis is an autonomous process by which excessive or potentially injurious cells are eliminated in multicellular organisms (Jacobson et al., 1997). Apoptotic cells are characterized by condensation of nuclear chromatin, DNA fragmentation (Earnshaw, 1995) the development of compact cytoplasmic organelles, and alterations in the plasma membrane (Arends and Wyllie, 1991). These events occur by distinct apoptotic pathways. One example is a family of cysteine proteases, caspases, which cleave their target proteins at specific aspartic acid sites (Alnemri et al., 1996). An apoptosis-inducing signal activates initiator caspases, which then activate effector caspases that inactivate apoptosis inhibitors, and alter homeostatic processes (Thornberry, 1999). Caspase-3 (CPP32, apopain) is a frequently evaluated effector caspase as studies have shown that it initiates DNA fragmentation (Wolf et al., 1999).

The present study evaluated Mn toxicity in vitro in two cell lines, CATH.a and SK-N-SH cells. CATH.a cells are an immortalized dopamine-producing cell line, while SK-N-SH cells are a non-catecholamine producing line.

MATERIALS AND METHODS

Tissue Culture

CATH.a cells were a generous gift from Dr. Dona Chikaraishi (Duke University, Durham, NC). The cells were grown in 75 mm^2 culture flasks at 37 °C in a humidified atmosphere (5% CO_2 and 95% air) in RPMI-1640 media supplemented with 8% horse serum, and 4% fetal calf serum. The cells were passaged twice weekly with 0.12% trypsin in PBS without ethylenediamine tetraacetic acid (EDTA). SK-N-SH cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and grown as previously described (Stokes et al., 2000, 2002).

Cytotoxicity Assay

Cell viability was determined using a high-throughput colorimetric assay (Cell Titer 96, Promega, Madison, MI). Viability was visualized by the conversion of a tetrazolium (Owen's reagent) compound to formazan. The cells used for the viability assay were seeded in polystyrene 96-well plates at a density of 30,000 cells per well and allowed to attach to the plate for 24 h. Cells were treated with varying concentrations of $MnCl_2$ and other test compounds for 24 h. Concentrations of Mn were selected based on a non-human primate study by Suzuki and colleagues showing central nervous system concentrations ranging from 35.3 to 334.4 μM Mn after 3 months exposure to manganese dioxide (Suzuki et al., 1975). Owen's reagent was incubated for an additional 2 h and absorbance at 490 nm was determined in a plate reader (Molecular Devices Max, Sunnyvale, CA). The quantity of

formazan product measured by the amount absorbed at 490 nm is directly proportional to the number of viable cells in culture. This relationship was confirmed in Trypan blue exclusion experiments (data not shown).

Catecholamine Quantification

CATH.a cells were seeded in 100 mm plates at a density of 8×10^6 cells per plate. To block DA synthesis, cells were treated, in triplicate with α-methyl-*para*-tyrosine (AMPT). The cells were exposed to AMPT (control, 10, 33, and 100 μM) for 24 h, followed by removal of the media and three rinses with phosphate buffered saline (PBS). Cellular DA content was determined by sonicating cells on ice in 0.3 ml of 0.2N perchloric acid, containing 1 μM 3,4-dihydroxybenzylamine as an internal standard and centrifuged at $10{,}000 \times g$ for 15 min. DA concentration in the resulting supernatant was analyzed by high pressure liquid chromatography (HPLC). 10 μl of supernatant was injected using a temperature controlled (4 °C) Waters 717 Plus Autosampler (Waters Corporation, Milford, MA) connected to a Waters 515 HPLC pump. Catecholamines were resolved on a reversed phase C18 column (Waters Symmetry, 4.6 mm × 250 mm, 5 μm, 100 A) and subsequently detected using a Waters 464 pulsed electrochemical detector (range 10 nA, potential 700 mV) connected by means of the Waters bus SAT/IN module to a computer using Millenium Software 32. The mobile phase consisted of 75 mM sodium dihydrogen phosphate, 1.7 mM 1-octanesulfonic acid, 25 μM EDTA, 10% v/v acetonitrile, all adjusted to a pH of 3.0 with phosphoric acid, pumped at a flow rate of 1 ml/min. Under these conditions, the average run time is 30 min with representative retention times (in min) for 3,4-dihydoxybenzylamine (internal standard, 8.24), and DA (11.28). Quantification was accomplished by the use of the internal standard method (10 pmol DHBA per injection) using daily standard curves of each analyte (0.5–25 pmol per injection). Results were expressed as μg protein/wet tissue weight. The limit of detection was 0.5 pmol per injection and inter assay variation was ±3%.

Intracellular GSH Measurements

CATH.a cells were seeded at a density of 5×10^6 cells per 100 mm dish. The cells were treated for 24 h in the presence of 5 mM glutathione (GSH), or 10 mM *N*-acetyl cysteine (NAC). Cells were washed in PBS, scraped, and harvested by centrifugation. After centrifugation and removal of the supernatant, 400 μl PBS was added to the cell pellet and samples were deproteinated by addition of 80 μl of 70% (v/v) perchloric acid. Following the addition of bathophenanthroline disulfonate and L-γ-glutamyl-L-glutamate, the mixture was vortexed and centrifuged and an aliquot was removed for derivation with iodoacetate and 1-fluoro-2,4-dinitrobenzene for HPLC analysis (Fariss and Reed, 1987). Separation of the resulting *S*-carboxymethyl-*N*-dinitrophenyl derivative of GSH was achieved with a reverse-phase, μBondapak amine 10 mm cartridge (8 mm × 10 cm) (Waters Associates, Milford, MA) with a Waters model 600E multisolvent delivery system using an ion-exchange method with a methanol–acetate mobile phase and gradient elution. Detection was performed at 365 nm and quantitation was derived from known standards using a Waters model 745B data module. To control for variations in sample volumes, peak areas were normalized to the internal standard. The limit of detection was approximately 50 pmol, which equated to approximately 0.4 nmol GSH/mg protein.

DNA Fragmentation Assay

CATH.a cells were seeded in 100 mm dishes at a density of 10×10^6–15×10^6 cells per dish. The cells were treated with varying concentrations of $MnCl_2$ for 24 h. One plate was treated for 3 h with 1 μM staurosporin (an antibiotic that inhibits phospholipid/calcium-dependent protein kinase and is a known inducer of apoptosis) as a positive control. After treatment, the cells were collected by centrifugation and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5, 40 mM EDTA, 0.5% Triton X-100) and incubated for 15 min at room temperature. The cells were then serially incubated with 50 μg/ml RNase A (Novagen, Madison, WI) for 1 h at 50 °C and with 0.5 mg/ml proteinase K (Promega, Madison, WI) at 50 °C for 1 h. The samples were extracted with an equal volume of H_2O-saturated phenol and twice with chloroform. 3.0 M sodium acetate/75% ethanol was added to the supernatant. DNA was precipitated overnight at −20 °C, collected by centrifugation, and resuspended in distilled water. Aliquots of DNA were resolved in a 1.5% agarose gel containing ethidium bromide and were visualized by UV transillumination.

Western Blot Analysis of Cleaved Caspase-3

CATH.a cells were seeded in 100 mm dishes at 8×10^6 cells per plate and allowed to adhere overnight. The following day the cells were exposed to 50 μM Mn

for 0, 3, 6, 12, 18, and 24 h. Cells were lysed using cell extraction buffer as directed by Cell Signaling Technology (Beverly, MA). The resulting supernatant was saved and assayed to determine the total protein concentration according to Bradford (1976) and 30 μg was electrophoresed on a denaturing SDS–polyacrylamide gel (SDS–PAGE) as described by Laemmli (1970). The protein was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) with a semi-dry apparatus (Hoeffer Semi Phor) at 75 mA for 75 min. After the transfer, cleaved caspase-3 proteins were detected with a rabbit anti-mouse polyclonal antibody diluted 1:1000 (Cell Signaling Technology, Beverly, MA) in 5% evaporated milk in PBS. Protein signals were visualized using a donkey anti-rabbit IgG diluted 1:2000 coupled with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) and SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Chemical, Rockford, IL). Independent samples were analyzed on a single Western blot, and the immunoreactive signals were quantified by transmissive densitometry (TINA, Fuji Medical, Stamford, CA).

RNA Isolation and Preparation

CATH.a cells (7×10^6) were plated in 100 mm dishes (Fisher Scientific Co., Pittsburgh, PA) and allowed to attach overnight. Mn (33 or 333 μM) was prepared in RPMI-1640 media and added for the specified time (3 or 12 h). Total RNA was extracted by the guanidine isothiocyanate method (TRI REAGENT, Molecular Reasearch Center Inc., Cincinnati, Ohio). Isolated RNA quantity and quality was assessed by spectrophotometry and denaturing electrophoresis, respectively.

Hybridization Array Analysis

RNA was isolated from seven plates each of control, 33 and 333 μM Mn-treated CATH.a cells. Equal amounts of RNA from each plate in the three groups were pooled to create three RNA pools. Each pool was treated with 5 μl DNase I for 30 min at 37 °C (1 mg/ml Boehringer Mannheim GmBH, Mannheim, Germany). ^{32}P-labeled cDNA probes were synthesized by reverse transcription of 5 μg total RNA from each pool as previously described (Freeman et al., 2001; Stokes et al., 2002). The array analysis was performed using mouse 1.2 Atlas® cDNA arrays according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). Equivalent amounts of radioactivity (approximately 5×10^6 cpm) were hybridized to Atlas® mouse 1.2 arrays (Clontech) overnight at 68 °C. The arrays were washed three times at 68 °C in 2× saline sodium citrate (SSC; 0.3 M NaCl/0.03 M sodium citrate) and 1% sodium dodecyl sulfate (SDS), followed by three additional washes at 68 °C in 0.1× SSC, 0.5% SDS. The radioactive signals were detected using a phosphorimager (BAS-5000, Fuji Medical, Stamford, CT).

Statistical Analysis

Values are expressed as mean ± S.E.M. unless otherwise stated. When comparing more than two groups, a one-way analysis of variance (ANOVA) was performed with the GraphPad In Stat statistical program. When the overall test of significance led to a rejection of the null hypothesis, a Tukey's multiple comparison post-test was performed. Analysis was carried out with the alpha level set at $P < 0.05$.

RESULTS

The effect of manganese on cell viability was compared in a catecholaminergic cell line (CATH.a) and a non-catecholamine-producing cell line (SK-N-SH), using a colorimetric assay. Mn was significantly ($P < 0.001$) more toxic to the catecholaminergic cells ($EC_{50} = 60$ μM) than the neuroblastoma cells ($EC_{50} = 200$ μM) (Fig. 1).

To test if DA is responsible for the heightened sensitivity of CATH.a cells to Mn toxicity, cellular DA concentrations in CATH.a cells were decreased by pretreating the cells with AMPT prior to Mn. AMPT decreases the catecholamine concentration in cells by inhibiting TH, the rate-limiting enzyme in catecholamine biosynthesis. Cells pretreated with 100 μM AMPT for 24 h had a 70% decrease in DA concentrations compared to control cells (Table 1). However,

Table 1
Catecholamine measurements in AMPT treated CATH.a cells

Treatment	Average (pmol/μg protein)	S.E.M.
Control	25.56	5
10 μM AMPT	25.87	3.73
33 μM AMPT	19.47	0.005
100 μM AMPT	8.24*	0.64

Dopamine concentrations were measured in CATH.a cells exposed to varying AMPT concentration for 24 h. Dopamine stores were decreased 70% with 100 μM AMPT.
* $P < 0.05$.

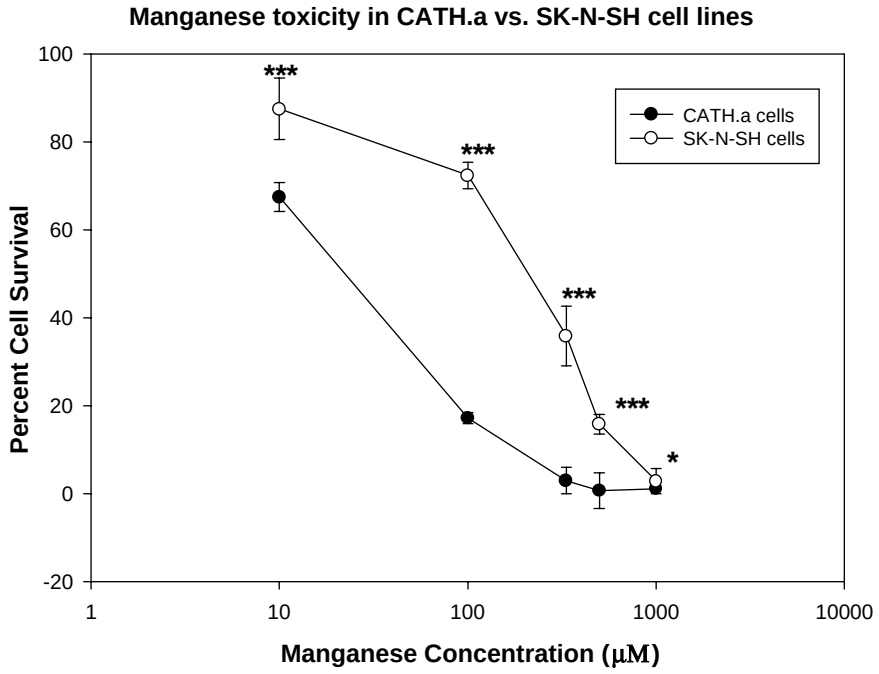

Fig. 1. Mn toxicity in CATH.a and SK-N-SH cell lines. Graph depicting cell death as percent of survival at increasing concentrations of Mn. SK-N-SH cells were resilient to the effects of Mn treatment when compared to CATH.a cells. Asterisks indicate significant difference ($^{***}P < 0.001$, $^{*}P < 0.05$).

depletion of DA in the CATH.a cells did not decrease the toxicity of Mn ($EC_{50} = 60$ μM) (Fig. 2), and cell viability remained unchanged compared to non-AMPT treated controls.

Past studies have suggested that Mn toxicity may, in part, be due to its ability to increase intracellular ROS formation and decrease antioxidant concentrations. To investigate a possible role of antioxidants in the

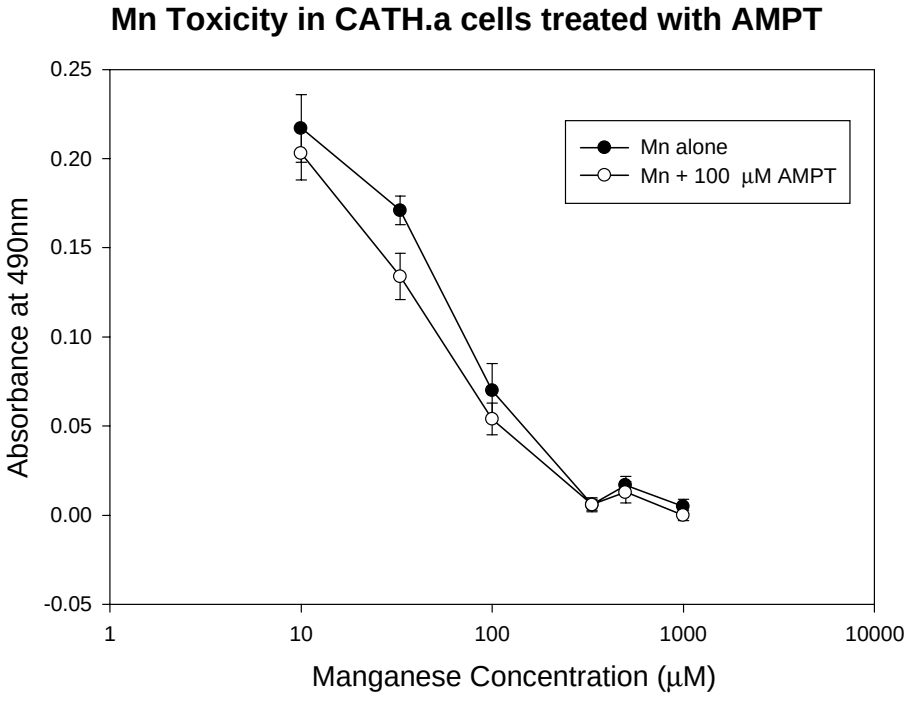

Fig. 2. Mn toxicity in presence of AMPT. Graph showing the absorbance of formazan, a product of viable cells in this assay, at increasing concentrations of Mn. CATH.a cells were treated with 100 μM AMPT for 24 h, then subject to cytotoxicity assay in conjunction with untreated cells. There were no significant differences at any treatment concentration.

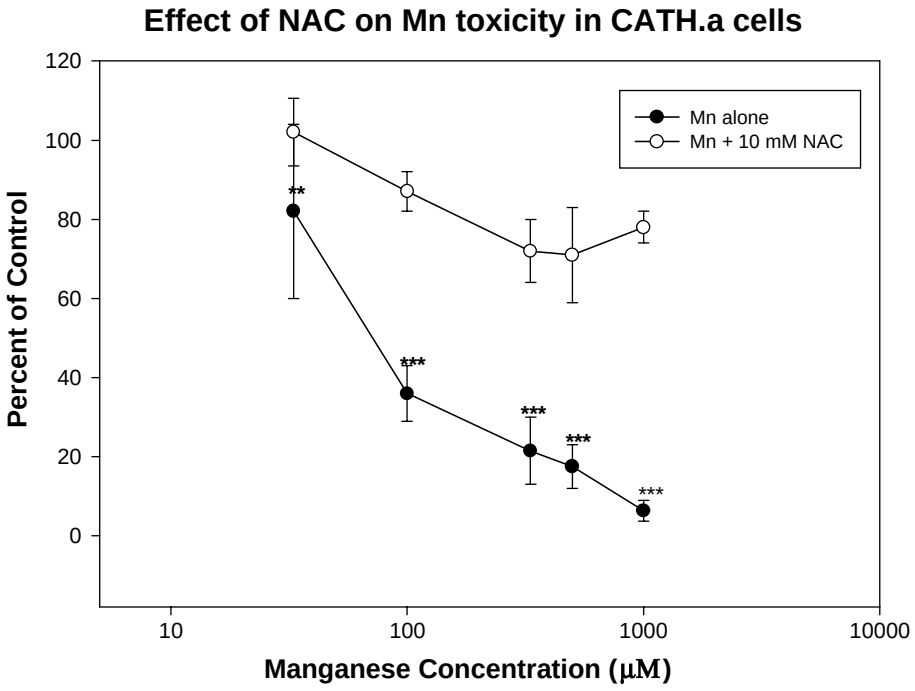

Fig. 3. Effect of NAC treatment on Mn toxicity in CATH.a cells. Graph depicting cell death as percent of control at increasing Mn concentrations. CATH.a cells underwent treatment with 10 mM NAC for 24 h or no treatment, followed by measurement of cell death. NAC treatment provided concentration-dependent protection from Mn at all concentrations tested. Significance is indicated by asterisks ($^{***}P < 0.001$, $^{*}P < 0.05$).

protection of CATH.a cells from Mn toxicity, the ability of two antioxidants, GSH (5 mM) and NAC (10 mM) to attenuate Mn toxicity was examined. A significant reduction in toxicity was observed with NAC (Fig. 3) and with GSH (Fig. 4) across the entire range of Mn concentrations. Finally, intracellular GSH levels were compared in control SK-N-SH cells (Stokes et al., 2000) and CATH.a cells to determine if differential levels of this antioxidant might account for the differential sensitivity to Mn. These studies indicated that the SK-N-SH cells (42.3 nmol GSH/mg cell pellet $\pm$ 10.5) had approximately three times higher intracellular GSH concentrations than the CATH.a cells (15.6 $\pm$ 1.6) ($P < 0.03$).

To test if GSH protection of CATH.a cells was mediated by blocking intracellular uptake of Mn into the cells, radiolabeled Mn uptake studies were performed. CATH.a cells were treated with Mn for 24 h after which time the 5 min uptake of ^{54}Mn was determined. No difference was observed in the uptake of Mn in cells treated with Mn versus cells treated GSH plus Mn (data not shown), suggesting that GSH does not protect cells from Mn by reducing its intracellular transport.

Because exogenous GSH does not readily cross cellular membranes, studies were performed to elaborate the mechanism of its protection against Mn toxicity. To examine the effect of GSH and NAC treatments on intracellular GSH levels, three treatment groups (control, 5 mM GSH, 10 mM NAC) were analyzed for GSH levels. Treatment with GSH and NAC, increased glutathione levels 6–7-fold times those levels found in the control cells (Table 2) ($P < 0.001$).

In order to further elucidate the mechanism of Mn toxicity, CATH.a cells were examined for evidence of internucleosomal DNA fragmentation. Cells were exposed to increasing concentrations of Mn (0–500 μM) for 24 h or 1 μM staurosporin for 3 h (positive control). Obvious internucleosomal fragmentation (an indicator of apoptosis) was detected in the positive control, but was only faintly evident in Mn-treated cells (data not shown). These data suggest that apoptosis likely does not play a role in Mn-induced cell death.

DNA fragmentation acts as the downstream result of apoptosis, but apoptosis is regulated by a variety of mechanisms, notably the caspase enzymes, in general, and caspase-3 in particular. Activation of caspase-3 requires proteolytic cleavage of its inactive zymogen into activated subunits. Using an antibody that detects the large fragment of active caspase-3 (17–20 kDa), it was found that 50 μM Mn (a toxic concentration) did not induce caspase-3 activation at 0, 3, 6, 12, 18 h, but caused a slight, yet insignificant induction at 24 h (data not shown). These data suggest that apoptosis may play

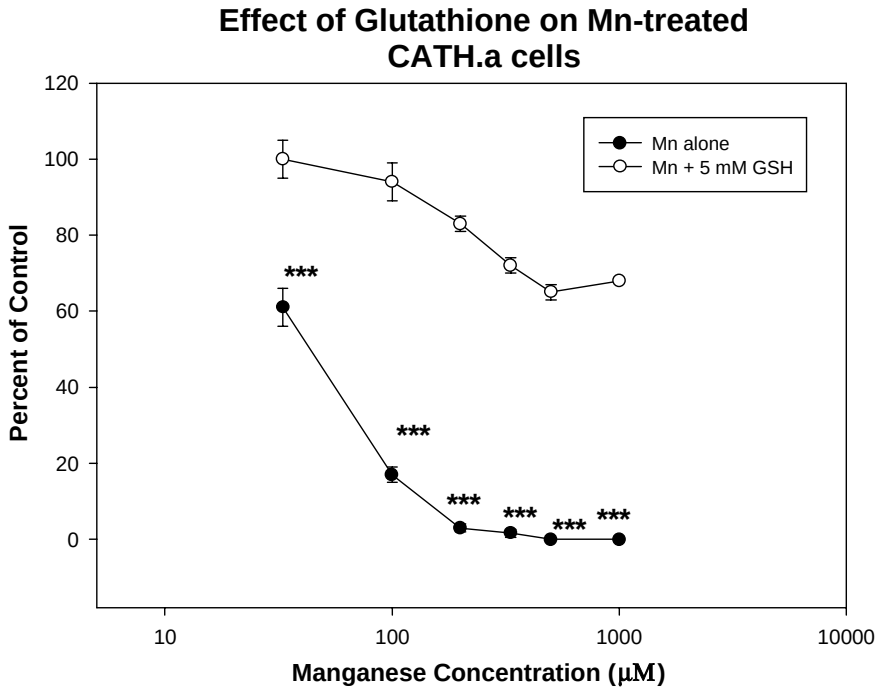

Fig. 4. Mn toxicity in CATH.a cells and attenuation with GSH. Graph depicting cell death as percent of control at increasing Mn concentrations. CATH.a cells underwent treatment with 5 mM GSH for 24 h or no treatment, followed by measurement of cell death. GSH treatment provided concentration-dependent protection from Mn at all concentrations tested. Significance is indicated by asterisks ($^{***}P < 0.001$).

a late role in cell death, leading to investigation of apoptosis-related genes.

Hybridization array analysis was performed to compare the effect of two different treatment groups (33 μM Mn for 3 h as well as a more toxic 333 μM Mn for 12 h) compared to their respective controls. The cDNA hybridization arrays (Atlas mouse 1.2 arrays; Clontech laboratories) contain 1176 gene-specific probes spotted in duplicate that are involved in a variety of functions including heat shock proteins, growth factors, cytokines, and transcription factors. This experimental approach is detailed in a review article published elsewhere (Vrana et al., 2003). Of particular interest for these studies were the apoptosis-related genes and regulating receptors. Previous studies in this laboratory have established that we can reliably detect changes of greater than 50% or reductions greater than 33% (Freeman et al., 2001; Stokes et al., 2002). The expression histograms shown in Fig. 5 represent genes found to be upregulated or downregulated in three sets of arrays for each treatment group. No significant changes were observed in any apoptosis-related genes in either treatment group (in three independent experiments). In the cells treated with 333 μM for 12 h, three housekeeping genes were nearly upregulated, while 78-kDa glucose regulated protein (GRP78) was the only gene found to be upregulated by 50%.

Table 2
Intracellular glutathione levels following GSH and NAC treatments

Treatment	GSH (nmol/mg)	S.E.M.
Control	15.6	1.7
5 mM GSH	102.5**	6.7
10 mM NAC	95.6**	1.8

CATH.a cells were plated at 6×10^6 cells per 100 mm dish. After allowing the cells to attach, the cells were exposed to GSH or NAC for 24 h. The homogenates were harvested and the glutathione levels were measured. The glutathione levels are 6–7 times higher in the GSH and NAC treated cells than in control cells.

** $P < 0.001$.

DISCUSSION

Mn toxicity has been studied since it was first described by Couper, who detailed Parkinsonian-like symptoms in five patients working in a Mn ore plant (Couper, 1837). Although Mn toxicity has been recognized for some time, the primary mechanism of its toxicity remains elusive, in spite of the fact that the Parkinson's disease-like symptoms suggest the involvement of the neurotransmitter DA (Barbeau et al., 1976; Newland et al., 1987).

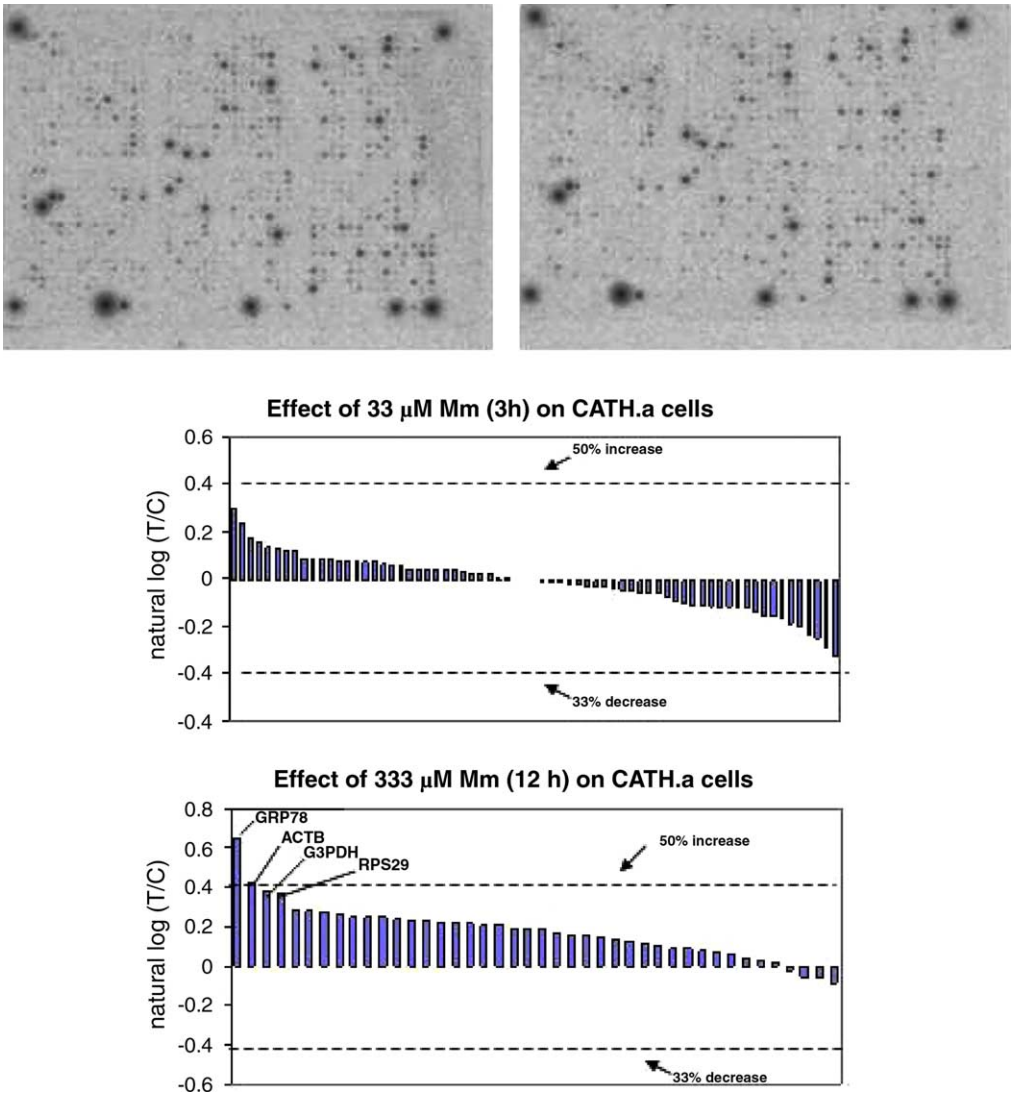

Fig. 5. Hybridization array analysis of Mn-induced gene expression of apoptosis-related genes. Two representative macroarrays at the 33 μM Mn concentration are preceded in the upper panels. Two expression histograms, the upper representing a 3 h exposure of CATH.a cells to 33 μM Mn and the lower a 12 h exposure to 333 μM Mn. Gene expression is plotted as the natural log of the treated vs. control ratio, which provides an equal scale for both up- and down-regulation. The 33 μM Mn concentration shows no robust changes in expression, while the 333 μM Mn concentration provides five up-regulated genes, none of which are involved in apoptosis. Similar results were obtained in two additional independent comparisons.

In the present studies, the toxicity of Mn was compared in a cell line that produces DA (CATH.a) with a SK-N-SH. Early results demonstrated that Mn is significantly more toxic to the catecholaminergic CATH.a cells ($EC_{50} = 60$ μM Mn) than to the SK-N-SH cells ($EC_{50} = 200$ μM Mn), raising the possibility that the presence of DA augmented Mn toxicity. The previous conclusion was reinforced by other studies that demonstrated a synergistic effect with Mn and DA in SK-N-SH cells (Stokes et al., 2000). No toxicity was observed in SK-N-SH cells treated with 200 μM DA or with 30 μM Mn. However, when the cells were exposed to subtoxic concentrations of DA and Mn simultaneously, a synergistic toxicity was demonstrated.

In an attempt to further identify whether DA is a mediator of Mn toxicity, cell viability in CATH.a cells was examined after DA depletion with AMPT. A 70% reduction in DA did not reduce Mn toxicity. Of interest is the fact that studies performed with AMPT concentrations as high as 300 μM (higher concentrations were themselves cytotoxic) displayed no decrease in Mn toxicity. This suggests that the heightened sensitivity of CATH.a cells versus SK-N-SH cells may not directly related to DA content, and that an alternative explanation may account for their heightened sensitivity to Mn.

Several studies have suggested that Mn toxicity is due to its ability to increase ROS formation (Graham, 1984) and decrease cellular protective mechanisms

(Desole et al., 1995; Graham, 1978; Liccione and Maines, 1988). In order to characterize the ability of antioxidants to afford protection from Mn toxicity, the effectiveness of GSH and NAC was studied in Mn-treated CATH.a cells. Protection from cell death was observed in cells treated with the antioxidants NAC (increases intracellular GSH) and GSH, suggesting a role for the formation of ROS in Mn neurotoxicity in CATH.a cells. This protection may work by offsetting the decreased levels of GSH observed in Mn-treated cells (Archibald and Tyree, 1987; Liccione and Maines, 1988). GSH does not readily enter cells, yet protected cells against Mn toxicity. To test if Mn uptake was being inhibited by GSH, the uptake of radiolabeled Mn into CATH.a cells treated with Mn/GSH and Mn alone was examined. No differences in Mn uptake were observed in Mn-treated cells versus control cells, suggesting that GSH did not protect cells by blocking intracellular uptake of Mn. Next, GSH and NAC-treated cells were analyzed for their GSH content compared to control cells. Both GSH and NAC caused a 6- to 7-fold increase in the antioxidant levels compared to control cells, and this effect was associated with attenuated Mn-induced cell death. The increase in intracellular GSH via exogenous GSH may be due to the presence of gamma-glutamyltranspeptidase (GGT). GGT is an enzyme that is located on the external part of the cellular membrane and is important in maintaining high intracellular GSH levels (Griffith et al., 1978). GGT cleaves GSH extracellularly to its constituent amino acids that can be absorbed into the cells. Once inside the cells, GSH is re-formed, using the amino acids that are transported in by the GGT (Griffith et al., 1978). Because of the existence of this biochemical mechanism, it is possible that the exogenous GSH is merely serving as a source of precursor molecules for the intracellular de novo synthesis of GSH. Since CATH.a cells are a relatively novel cell line, the presence of GGT in this line has yet to be established and warrants further examination.

These data suggest the following: Mn toxicity may involve the formation of ROS, protection from Mn by NAC and GSH does not occur by preventing the entry of Mn into the cells, and the cells take up GSH, presumably due to the presence of GGT. Importantly, increased GSH levels are associated with reduced Mn-induced cell death; lending further credence to the hypothesis that Mn-induced cell death is a consequence of ROS formation. Furthermore, comparison of intracellular GSH levels in non-treated SK-N-SH cells (Stokes et al., 2000) and CATH.a cells indicated that the former have approximately three times more intracellular GSH than the latter cells. Again, indicating a role for ROS in Mn-induced cell toxicity.

Mn toxicity has been reported to work through apoptotic mechanisms (Desole et al., 1996; Schrantz et al., 1999). A number of apoptotic markers exist including DNA fragmentation and increased gene expression of apoptotic pathways. Cells exposed to varying concentrations of Mn for 24 h presented only marginal DNA laddering. Caspases are a group of inactive proenzymes that are activated by cleavage at specific aspartate sites and are involved in apoptosis. One prominent caspase is capase-3, which is activated by proteolytic cleavage yielding two fragments (Nicholson et al., 1995). Immunoblot analysis of the large caspase-3 fragment in cells exposed to 50 μM Mn (a concentration with minor DNA laddering) from time points ranging from 3 to 18 h showed no sign of caspase-3 activity, while a small amount of activity was shown at 24 h. The inconclusiveness of the apoptotic markers, DNA laddering and caspase-3, led to the employment of hybridization array technology for the analysis of the effect of Mn on apoptosis-related genes (reviewed in Vrana et al., 2003). The effects of two different concentrations of Mn were tested on gene expression with the use of hybridization array technology. The first set of arrays ($n = 3$) was performed with low concentrations of Mn (33 μM) at a relatively short period of time (3 h). This was intended to provide insight into acute effects of Mn on gene expression. The second set of arrays ($n = 3$) was performed using higher concentrations of Mn (333 μM) for a longer period of time (12 h). Importantly, these timepoints are before large-scale cell death, meaning that changes in gene expression are possible causative events in cell death and not just indicative of a breakdown in cellular machinery. The studies were performed to identify changes (possibly in the expression of apoptotic genes) that occur before the cells' demise. No changes in apoptosis-related genes were seen. Of the 40 apoptosis-related genes present on the array only 3 genes were even detected as present (RAD21 homolog, RAD23 UV excision repair protein homolog, and defender against cell death (DAD)). The possibility remains that apoptotic genes are induced by Mn, but through translational rather than transcriptional mechanisms. Mn-induced apoptosis was not seen with any of the traditional markers examined in the study, but cell death may also occur by a combination of events (involving both gene programmed events and necrosis). Changes in four genes were observed with the hybridization

arrays; 78-kDa GRP78, cytoplasmic beta-actin (ACTB), glyceraldehyde-3 phosphate dehydrogenase (G3PDH), and 40S ribosomal protein S29 (RPS29) were all induced by Mn treatment. It is important to note that GRP78 met the threshold criteria for significance, while the other three nearly reached that arbitrary level of change.

In summary, the present study points to the formation of ROS as the primary mechanism for Mn-induced toxicity in CATH.a cells, although a DA-dependent mechanism may still be involved even with decreased DA levels. The increased sensitivity to Mn of CATH.a cells compared to SK-N-SH cells is likely due to lower GSH levels in the CATH.a cells. The protection from Mn observed with the addition of GSH and NAC suggests that Mn-induced cytotoxicity is due to ROS formation. Future studies are needed to substantiate ROS formation as the primary mechanism for Mn toxicity. Depletion of GSH by buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis on Mn toxicity, should be performed to see if decreasing GSH levels in cells increases Mn toxicity. Also direct measurement of Mn-induced ROS formation should be performed. Combined, those studies should firmly establish a primary role for ROS formation in Mn toxicity.

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