Manganese Upregulates Cellular Prion Protein and Contributes to Altered Stabilization and Proteolysis: Relevance to Role of Metals in Pathogenesis of Prion Disease

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Prion diseases are fatal neurodegenerative diseases resulting from misfolding of normal cellular prion (PrPC) into an abnormal form of scrapie prion (PrPSc). The cellular mechanisms underlying the misfolding of PrPC are not well understood. Since cellular prion proteins harbor divalent metal-binding sites in the N-terminal region, we examined the effect of manganese on PrPC processing in in vitro models of prion disease. Exposure to manganese significantly increased PrPC levels both in cytosolic and in membrane-rich fractions in a time-dependent manner. Manganese-induced PrPC upregulation was independent of messenger RNA transcription or stability. Additionally, manganese treatment did not alter the PrPC degradation by either proteasomal or lysosomal pathways. Interestingly, pulse-chase analysis showed that the PrPC turnover rate was significantly altered with manganese treatment, indicating increased stability of PrPC with the metal exposure. Limited proteolysis studies with proteinase-K further supported that manganese increases the stability of PrPC. Incubation of mouse brain slice cultures with manganese also resulted in increased prion protein levels and higher intracellular manganese accumulation. Furthermore, exposure of manganese to an infectious prion cell model, mouse Rocky Mountain Laboratory–infected CAD5 cells, significantly increased prion protein levels. Collectively, our results demonstrate for the first time that divalent metal manganese can alter the stability of prion proteins and suggest that manganese-induced stabilization of prion protein may play a role in prion protein misfolding and prion disease pathogenesis.

Key Words: metals; neurotoxicity; manganese; scrapie; environmental factors; prion accumulation.

Prion diseases are fatal neurodegenerative disorders affecting humans (Creutzfeldt-Jakob disease), cattle (bovine spongiform encephalopathy [BSE]), deer (chronic wasting disease), goat, and sheep (scrapie). Regions of the brain that control motor function, including the basal ganglia, cerebral cortex, thalamus, brain stem, and cerebellum, are severely affected in prion diseases. The major neurological symptoms are extrapyramidal motor signs, including tremors, ataxia, and myoclonus (Aguzzi and Heikenwalder, 2006; Johnson, 2005). Neuropathological changes include vacuolation of neutrophils, neuronal loss, and gliosis in brains of diseased animals and humans (Collinge and Palmer, 1992; Palmer and Collinge, 1992). Once thought to be caused by a virus-like particle, prion diseases were later proven to be caused by an abnormal conformation of a host prion protein (Chatriy and Prusiner, 1980). Normal prion protein (PrPC) is a cell surface glycoprotein expressed predominantly in the central nervous system and converted to the proteinase-resistant aggregate form (PrPSc) during the disease state (Collinge, 2005; Prusiner and Kingsbury, 1985). Neuropathological characterization of prion disease involves massive neuronal degeneration associated with accumulation of the abnormal prion protein PrPSc derived from the normal prion protein PrPC (Collinge, 2005; Ma and Lindquist, 2002). However, still the cellular and molecular mechanisms underlying the conversion of the normal form of PrPC into the proteinase-K (PK)–resistant diseased form PrPSc are yet to be identified.

Although normal cellular prion protein PrPC is highly expressed in the brain, the endogenous function of this protein has not been completely elucidated. PrPC has been suggested to function as an antioxidant, a cellular adhesion molecule, a signal transducer, and a metal-binding protein (Chiariini et al., 2002). While the function of PrPC is not well defined, the hallmark of prion disease is conversion of the soluble form of normal prion protein to the insoluble β-sheet-rich and infectious form through a still unknown mechanism (Prusiner and Kingsbury, 1985). PrPC is a cell surface protein linked via
a glycosyl phosphoinositol anchor, with two N-linked glycosylations, and a disulfide bridge. PrP<sup>C</sup> contains several octapeptide repeat sequences (PHGGSWGQ) toward the N-terminus that have high binding affinity for divalent metals, such as copper, manganese, and zinc, and preferential binding for copper (Cu) (Brown, 2009; Hornshaw et al., 1995). The antioxidant properties of PrP<sup>C</sup> have been linked to Cu residency in the octapeptide repeat domain (Brown, 2009). Loss of this antioxidant activity has been linked to neurodegeneration seen in prion disease. Furthermore, the metal-binding sites have been suggested to play a role in the pathogenesis of prion diseases (Brown, 2009; Hornshaw et al., 1995; Moore et al., 2006). PrP<sup>C</sup> is believed to play a role in iron homeostasis, and iron binding to prion protein apparently affects the conversion to a protease-resistant form of prion protein (Singh et al., 2009). Interestingly, altered manganese (Mn) content has been observed in the blood and brain of humans infected with the prion disease Creutzfeldt-Jakob Disease (CJD), in mice infected with scrapie, and in cattle infected with BSE (Brown, 2009). Additionally, manganese-bound PrP<sup>C</sup> can be isolated from both humans and animals infected with prion disease. Despite these findings, the role of manganese in the pathogenesis of prion disease is currently unknown.

Recently, we studied the effect of Mn on oxidative stress, mitochondrial function, cellular antioxidants, proteasomal function, and protein aggregation in cell culture models of prion diseases (Choi et al., 2006, 2007). We have demonstrated that normal prion protein reduces manganese transport and protects the cells from manganese-induced oxidative stress, mitochondrial dysfunction, cellular antioxidant depletion, and apoptosis, suggesting that normal cellular prion interacts with manganese and protects cells from manganese neurotoxicity at early stages of exposure (Choi et al., 2006, 2007). We also reported that PrP<sup>C</sup> protects against apoptotic cell death during oxidative stress but exacerbates apoptosis during endoplasmic reticular (ER) stress (Anantharam et al., 2008). While studying the role of prion protein in metal neurotoxicity, we unexpectedly found that manganese exposure upregulated cellular prion protein in neuronal cell models. Therefore, in the present study, we systematically characterized mechanisms underlying manganese-induced prion protein accumulation and its biological relevance using mouse cell culture models and brain slices.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Manganese chloride (MnCl<sub>2</sub>), MG-132, and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma (St Louis, MO); PMSF protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, IN); and Bradford Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA). Dulbecco’s modified Eagle medium (DMEM), Opti-MEM, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA); PK was purchased from Promega (Madison, WI). DRAQ5 nuclear stain was purchased from Alexis Biochemicals (San Diego, CA). ECL chemiluminescence kit and [35S]-methionine were purchased from Amersham (Piscataway, NJ). Monoclonal mouse anti-PrP (3F4) antibody recognizing sequences 109 through 112 in hamster and human PrP was purchased from Signet Labs (Berkeley, CA). Monoclonal mouse anti-PrP (SAF32) antibody recognizing murine PrP was purchased from Cayman Chemicals (Ann Arbor, MI). Antiubiquitin polyclonal antibody was purchased from DAKO Cytomation (Carpinteria, CA). β-actin antibody was purchased from Sigma.

**Cell culture.** Mouse neuronal cells expressing mouse prion protein with 3F4-hamster epitope (PrP<sup>F346</sup>), kindly provided by Dr Suzette Priola at Rocky Mountain Laboratory (NIAIDS, Hamilton, MT), were cultured as described previously (Anantharam et al., 2008; Choi et al., 2007). Cells at ~75% confluence were treated with varying concentrations of manganese (0, 100, 300, and 500μM) in T25 cell culture flasks and then collected for biochemical analyses. Cells were harvested into phosphate-buffered saline (PBS) and resuspended in either homogenization buffer (20mM Tris-HCl, pH 8.0, 2mM EDTA, 10mM ethylene glycol tetraacetic acid (EGTA), 2mM dithioreitol, 1mM PMSF, 25μg/ml aprotinin, and 10μg/ml leupeptin) or lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 5mM Tris-HCl, 150mM NaCl, and 5mM EDTA in PBS, pH 7.4). For preparation of soluble and insoluble cell fractions, low-detergent lysis buffer (20mM Tris-HCl, pH 8.0, 10mM EGTA, 2mM EDTA, 2mM dithioreitol, 1mM phenylmethylsulfonylfluoride, 25μg/ml aprotinin, and 10μg/ml leupeptin) and insoluble lysis buffer (20mM Tris-HCl, pH 8.0, 10mM EGTA, 2mM EDTA, 2mM dithioreitol, 1mM phenylmethylsulfonylfluoride, 25μg/ml aprotinin, 10μg/ml leupeptin, 0.5% Triton X-100, and 0.2% SDS) were used.

**Cell culture model of infectious prion disease.** Rocky Mountain Laboratory (RML) mouse scrapie-infected Cath. A-differentiated cells (CAD5) and uninfected CAD5 cells were kindly provided by Dr Charles Weissmann of Scripps Institute (Jupiter, FL). Cell culture conditions used in this study were similar to those described previously (Mahal et al., 2007). Briefly, 20,000 cells were seeded in a 96-well plate and allowed to grow for 16 h. Infection was performed by addition of 0.3 ml of 5 × 10<sup>6</sup> RML-infected brain homogenate in Opti-MEM supplemented with 10% FBS and 90 μl penicillin and 90 μl streptomycin per milliliter. After three 1:8 splits, the well with the highest amount of infected cells was subcloned and reinfected with 10<sup>-7</sup> RML-infected brain homogenate. The most positive clone from a 14 split was then expanded and frozen in 50% FBS, 10% DMSO, and 40% Opti-MEM. RML infection was periodically verified by PK-resistant prion protein analysis with Western blot.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting.** After harvest, cells were lysed, homogenized, sonicated, and centrifuged as described previously (Sun et al., 2005). The supernatants were collected from cell lysates, and protein concentrations were determined and used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cytoplasmic and membrane-rich fractions containing equal amounts of protein were loaded in each lane and separated on a 15% SDS-PAGE as described previously (Sun et al., 2005).

For determination of high–molecular weight ubiquitinated proteins, low-detergent soluble and insoluble fractions were separated according to a procedure described previously, with slight modifications (Sun et al., 2005). After exposure to manganese or proteasome inhibitor, MG-132 cells were collected and washed once with ice-cold PBS. The cell pellets were suspended in low-detergent lysis buffer. Lysates were ultracentrifuged at 100,000 × g for 40 min, and the detergent-soluble fraction was obtained by collecting the resulting supernatant. The detergent-insoluble pellets were washed once with lysis buffer and resuspended in insoluble lysis buffer and sonicated for 20 s. Equal amounts of protein were loaded in 8% SDS-PAGE, as determined by the Bradford protein assay. Proteins were then transferred to a nitrocellulose membrane, and nonglycosylated binding sites were blocked by treating with 5% nonfat dry milk powder. Membranes were then treated with primary antibody directed against the...
Limited proteolysis of prion protein. The relative resistance of prion protein to PK digestion was determined as previously described, with slight modifications (Prusiner and Farquhar, 1989). Cells were cultured with 100μM Mn, while untreated cells were used as the control. After a 24-h incubation period, cells were collected and washed once with PBS. Lysis buffer was added to the pellet and vortexed. Clarified cell lysates were obtained by centrifugation at 16,000 x g for 10 min. PK was added to the cell lysates at a final concentration of 1 μg/ml and incubated at 37°C for selected time points. To stop the digestion, PMSF was added to a final concentration of 4mM, and sample loading buffer was added to the digested lysates prior to analysis by Western blot. For cell-free studies, cells at confluency were collected and lysed in the lysis buffer. Following protein measurements using Bradford’s method, −5 mg/ml of cell lysates were separated into manganese-treated and untreated control groups. For manganese treatment, 100μM Mn was added to the cell lysate that was incubated at 37°C for 1 h prior to limited proteolysis.

Mouse brain slice preparation. Mouse brain slices were obtained from adult C57BL/6 mice. All experimental procedures were approved by the Animal Care and Use Committee at Iowa State University and were in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” Animals were anesthetized with isofluorane, and brain was removed and placed in ice-cold artificial cerebrospinal fluid (aCSF); 125mM NaCl, 2.5mM KCl, 26mM NaHCO3, 1.2mM NaH2PO4, 1.2mM MgSO4, 2mM CaCl2, and 15mM dextrose). Coronal slices (400 μm thick) were obtained and placed in ice-cold aCSF. After preparation, the slices were transferred to sterile porous membrane units (Millipore 12 mm CM PTFE 0.4 μm, Millipore, Billerica, MA) and preincubated in incubation media containing 25% Hank’s Balanced Salt Solution, 25% heat-inactivated horse serum, 50% Modified Eagle Medium, 5mM L-glutamine, 6.5 mg/ml D-glucose, 20mM HEPES, and streptomyccin/penicillin (5 μg/ml and 5000 U/ml, respectively) for 2 h prior to initiation of manganese treatments. Slices were cultured for 1, 3, and 7 days at 37°C in 5% CO2 with or without manganese (300μM). The culture medium was changed every 2 days until the last day of treatment.

Quantification of brain-derived PrP. Brain homogenates were prepared (10% wt/vol) in lysis buffer containing 150mM NaCl, 10mM Tris-HCl, 10mM EGTA, 2mM EDTA, and 1mM PMSF protease inhibitor cocktail. Protein concentration was measured using the Bradford method, as previously described (Choi et al., 2007). An equal concentration of protein was loaded for each sample, and separated proteins were transferred onto nitrocellulose membrane and probed with SAF32 monoclonal antibody. Following primary antibody incubation, the blots were washed thoroughly and incubated with horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence. For verification of equal loading of proteins, the blots were stripped and reprobed with β-actin. All images were captured with a Kodak 2000MM imaging system. PrP levels were normalized to β-actin and plotted as percentage of PrP level of untreated sample at day 1.

Measurement of manganese concentration in mouse brain slices. Brain slices were collected at 1, 3, and 7 days following treatment and weighed prior to analysis with inductively coupled plasma mass spectrometry (ICP-MS) to determine the concentrations of Mn, Fe, Cu, and Zn in each sample. The ICP-MS device was a high-resolution double-focusing instrument operated at medium resolution (m/Δm = 4000) in order to resolve the isotopes of interest from any interferences (Choi et al., 2007). The signals from the two most abundant isotopes of Fe, Cu, or Zn were measured, and the average concentration calculated from both isotopes is reported. Manganese is monoisotopic, so its concentration was calculated from the signal from one of the Mn isotopes.
isotope. Each sample was placed in an acid-washed 5 ml Teflon vial and digested in 1% high-purity nitric acid. Following digestion, the samples were diluted to 5 ml with Milli-Q 18.2 M deionized water for a final acid concentration of ~2% nitric acid. The supernatant was analyzed with ICP-MS. An internal standard method was used for quantification. Gallium was chosen as the internal standard because its m/z ratio is similar to that of the elements of interest, and it has no major spectroscopic interferences. A small spike of Ga standard solution was added to each sample such that the final concentration of Ga was 10 ppb. A 10 ppb multielement standard (Mn, Fe, Cu, Zn, and Ga) was also prepared. The nitric acid blank, the multielement standard, and each of the samples were introduced into the ICP-MS via a 100 μl/min self-aspirating PFA nebulizer (Elemental Scientific, Inc.). Between samples, the nitric acid blank was used to rinse the nebulizer. The results for each sample are calculated using the integrated average background-subtracted peak intensities from 20 consecutive scans. In order to correct for differences in isotopic abundance and elemental sensitivity in the ICP, the multielement standard was used to derive normalization factors for Mn, Fe, Cu, and Zn. The concentration of each element was then calculated for each sample.

Data analysis and statistics. Data were analyzed with Prism 4.0 software (GraphPad Software, San Diego, CA). Bonferroni post hoc multiple comparison testing was used to delineate significance between manganese-treated groups and the control (untreated) samples. Differences with p < 0.05 were considered significant and are indicated with asterisks. For densitometric analysis of limited proteolysis, band intensity was normalized to control bands at 0 min, and one-phase exponential decay was fit to the data.

RESULTS

Manganese Treatment Upregulates Normal Cellular Prion Protein (PrP<sup>C</sup>) Expression

Previously, we showed that the EC<sub>50</sub> of manganese was 100 μM in mouse PrP<sup>C</sup>-expressing cells (Choi et al., 2007), and this concentration was used for all experiments. First, we measured cellular prion protein (PrP<sup>C</sup>) levels in the cytoplasm

FIG. 1. Manganese-induced cellular prion protein (PrP<sup>C</sup>) upregulation in mouse neuronal cells. (A) Western blot analysis of PrP<sup>C</sup> at various time points following manganese treatment. The cells were treated with manganese (100 μM) for 0, 6, 12, 18, and 24 h and harvested. Cytosolic fractions and membrane-rich fractions were obtained and analyzed by Western blotting. (B) Densitometry analysis of PrP<sup>C</sup> bands (17–37 kDa) in (A). *p < 0.05 or **p < 0.01 compared with the control group. (C) ICC expression of PrP in cells with and without manganese treatment. PrP<sup>C</sup> staining is shown in green and nucleus staining is in blue.
and membrane following manganese treatment using Western blot. As shown in Figure 1A, manganese-treated cells showed a time-dependent increase in PrP C expression in both cytosolic and membrane fractions up to 24 h. The increase was observed as early as 6 h after manganese exposure. Untreated control cells did not exhibit any significant increase in PrP C levels. Densitometry analysis of prion protein bands (Fig. 1A) shows a time-dependent increase in prion protein levels over a 24-h time period in both cytosolic and membrane fractions (Fig. 1B). Equivalent loading of proteins in each lane was confirmed using β-actin as the internal control. To further characterize the cellular distribution of prion protein levels, the immunocytochemical (ICC) method was employed. Confocal image analysis of immunohistochemically processed samples revealed more accumulation of PrP C in both the plasma membrane and the cytoplasm of manganese-treated cells as compared to untreated cells (Fig. 1C). Manganese-treated cells at 18 h showed significant morphological changes, with stronger immunoreactivity of PrP C in cytosol and membrane and fewer neuronal processes as compared to the control cells. At earlier time points of 6–12 h, no significant morphological changes were noted in Mn-treated cells, but prion protein immunoreactivity was evident in both cytoplasm and plasma membrane (data not shown). Collectively, these results suggest that manganese exposure significantly upregulates prion protein levels in neuronal cells.

**Manganese-Induced PrP C Upregulation is not due to Increased Transcriptional Rate or Impaired Protein Degradation Machinery**

Next, we carried out a series of experiments to determine the cellular mechanisms underlying Mn-induced PrP C upregulation. We sought to examine whether the increase in PrP C level during Mn exposure is due to increase in transcription of PrP messenger RNA (mRNA) or due to impairment in degradation of PrP C protein by the ubiquitin proteasome system (UPS) or lysozymes. PrP mRNA expression in Mn-treated cells was measured by quantitative reverse-transcriptase polymerase
PrPC was immunoprecipitated with 3F4 antibody and then subjected to SDS-PAGE to confirm that the increased PrP C levels were not due to differences in labelling efficiency between manganese-treated and control cells. To further rule out possible involvement of another cellular degradative system during manganese treatment, lysosomal activity was measured. Lysosomal activity assay indicated that there was no significant difference between manganese-treated and control cells (Fig. 2E). Egg white lysozyme was used as a positive control for the lysosomal assay. These results collectively demonstrate that Mn-induced prion protein upregulation is not due to increased transcriptional rate, mRNA stability, or impairment of protein degradative machinery.

**Manganese Delays the Turnover Rate of PrP**

Since neither the transcriptional rate nor the protein degradation was altered, we proceeded to investigate whether manganese altered the cellular metabolism of PrP. We measured the effect of manganese on prion protein turnover rate in a pulse-chase experiment. The cells were labeled with [35S]-methionine and then exposed to 100µM Mn, and the prion protein turnover was chased at various time points for a total of 24 h posttreatment. As shown in Figure 3A, manganese treatment protracted the turnover rate of PrP as compared to the control group. The densitometric analysis of prion protein turnover in the autoradiogram was quantified at various chase time points (Fig. 3B). Manganese treatment significantly altered the turnover rate of prion protein over a 24-h chase period. The half-life of PrP in control samples was determined to be 13 h, while the half-life of PrP was increased to 21 h in manganese-treated samples. These data indicate that manganese treatment increases the stability of prion protein, resulting in higher accumulation of PrP in manganese-treated cells relative to controls.

**Manganese Treatment Decreases Proteolytic Susceptibility of PrP**

We next determined whether manganese treatment alters the proteolytic rate of prion proteins by performing a limited proteolysis assay with PK. PK-dependent proteolysis of prion protein is traditionally used for determination of the pathogenic form of PrPSc (Brown, 2009). Egg white lysozyme was used as a positive control for the lysosomal assay. These results suggest that the altered protease resistance of PrP in manganese-treated cell
samples is a result of manganese binding to prion protein, which stabilizes the prion protein inside the cell and confers higher PK resistance. Also shown in Figure 4B are the best-fit parameters of a single-phase exponential of data. The analysis revealed that $K_{\text{obs}}$ for PrP$^{C}$ was significantly reduced in manganese-treated cell lysates, whereas the PrP$^{C}$ half-life was significantly increased in manganese-treated cells as compared to untreated control cells. Half-life for PrP$^{C}$ proteolysis was determined to be 1.65 and 7.5 min for untreated and manganese-treated cells, respectively. However, the half-life for β-actin was unaltered: 0.84 and 0.83 min for untreated and manganese-treated cells, respectively.

**PrP Expression in Mouse Brain Slice Cultures Treated with Manganese**

In order to extend our studies to brain tissues, we examined the effect of manganese on prion protein in mouse brain slice culture models. Mouse brain slices were prepared and then exposed to 300 μM manganese for 1, 3, and 7 days. Following the treatment, tissues were lysed and equal amounts of protein were used for measurement of prion protein levels by Western blot analysis. As shown in Figure 5A, a time-dependent increase in PrP expression was observed in manganese-treated slices as compared to the control slices. All three isoforms, including di-glycosylated, mono-glycosylated, and unglycosylated isoforms, were increased. However, there was no significant change in the β-actin protein level, which was used as a loading control. Figure 5B shows the densitometric data for the 37 kDa band prion protein levels. These results show that manganese exposure significantly upregulates the level of prion protein in brain tissues, consistent with data obtained in cell culture models.

**Manganese Levels in Brain Slices**

To verify manganese uptake into the mouse brain slice cultures, slices were collected following treatment and processed for metal analysis by ICP-MS. As shown in Figure 6A, a significant increase in manganese content was observed in samples treated with manganese starting at day 1. The manganese uptake was quite evident as early as day 1 of treatment. However, levels of other divalent cations, copper (Fig. 6B), iron (Fig. 6C), and zinc (data not shown), were not
significantly altered by manganese treatment and remained unchanged through 7 days of manganese treatment. The ICP-MS data suggest increased intracellular Mn levels during chronic manganese exposure in brain slices.

**Manganese Induces Upregulation of Prion Protein in a Scrapie-Infected Cell Culture Model of Prion Disease**

Next, we examined whether Mn is capable of upregulating PrP levels in the infectious form of prion protein, PrP\textsuperscript{Sc}. We used the RML scrapie–infected Cath. A-differentiated (CAD5) mouse neuronal cell line for this study. This cell model of infectious prion disease was obtained from Dr Charles Weissmann, whose laboratory at Scripps Institute, Florida, recently demonstrated that RML-infected CAD5 cells make an excellent cell culture model of infectious prion disease because these cells propagate PrP\textsuperscript{Sc} infection through multiple passages without the need for reinfection (Mahal et al., 2007). We first demonstrate the presence of PK-resistant PrP\textsuperscript{Sc} prion protein in scrapie-infected CAD5 cells by performing a limited proteolysis assay with PK. Prion proteins were immunoprecipitated with 6H4 antibody from uninfected and scrapie-infected CAD5 cells and then the proteolytic susceptibility was monitored. As shown in Figure 7A, PK-resistant PrP\textsuperscript{Sc} protein was present in scrapie-infected CAD5 immunoprecipitates but not in uninfected CAD5 immunoprecipitates. RML scrapie–infected and uninfected CAD5 cells were exposed to 200 \(\mu\)M Mn for 12 h. Cells were harvested and subjected to Western blot analysis for PrP protein expression levels. As shown in Figure 7B, manganese induced similar increases in PrP protein levels in both uninfected and RML scrapie-infected CAD5 cells, suggesting that manganese treatment can affect PrP expression even during the progression of prion disease in infected cell culture models.

**DISCUSSION**

Our results demonstrate that manganese exposure upregulates prion protein levels without altering transcription of PrP mRNA or degradation of the protein. The increased protein level is mainly attributed to stabilization of the protein, as determined by increased half-life of PrP\textsuperscript{C} in a pulse-chase experiment. In this study, we show that manganese-induced prion protein upregulation in various models, including neuronal cell cultures, mouse brain slices, and an infectious cell culture model of prion disease, confirming the reproducibility and biological significance of our novel observation. Our study also demonstrates that manganese treatment increases the

**FIG. 5.** Manganese-induced PrP\textsuperscript{C} upregulation in mouse brain slices. Mouse brain slice cultures were exposed to 300\(\mu\)M manganese for 1, 3, and 7 days, and slices were then homogenized and subjected to Western blot and metal analysis. (A) Representative Western blot analysis of total PrP\textsuperscript{C} levels in mouse brain slices treated with manganese and in untreated slices is shown at top. (B) Below, the representative Western blot image is the quantification of band intensity of PrP\textsuperscript{C} normalized with β-actin levels. Each data point represents experiments performed in triplicate *\(p < 0.05\).

**FIG. 6.** Divalent cation levels in mouse brain slices following manganese treatment. Quantitative analysis of divalent cation levels in mouse brain slice culture treated with 300\(\mu\)M manganese for 1, 3, and 7 days is shown in (A) Manganese, (B) Copper, and (C) Iron. Each data point represents experiments normalized to wet weight of mouse brain slices performed in triplicate ***\(p < 0.001\).
PrP<sup>C</sup> resistance to PK-dependent proteolysis similar to that observed for scrapie prion protein (PrP<sup>Sc</sup>). Additionally, results with an infected cell culture model reveal that manganese can increase the upregulation of the infectious form of prion protein, indicating the importance of metals in prion pathogenesis. To our knowledge, this is the first time that upregulation of prion protein independent of transcription contributing to enhanced stabilization and resistance to proteolysis is demonstrated. Thus, our results of manganese-induced stabilization of prion protein suggest that altered metal homeostasis may play an important role in the pathogenesis of prion diseases.

Many physiological and cellular changes occur during prion infections of central nervous system, such as neuronal vacuolization, neuronal loss, gliosis, oxidative impairment, and metal imbalance (Brown, 2009). Some of the most striking changes at the cellular level after prion infection are the loss of antioxidant function and altered metal content, suggesting a role of metals in the pathogenesis of prion diseases (Brown, 2009). In particular, increased manganese in the brains of various prion diseases, including BSE and CJD, were reported (Brown, 2009). We recently showed that cellular prion protein protects against oxidative stress–induced apoptotic cell death (Anantharam et al., 2008). We also demonstrated that manganese binds to prion protein and reduces the manganese transport into cells (Choi et al., 2007). We reported that PrP<sup>C</sup> expressing cells were more resistant to manganese-induced cytotoxic and apoptotic cell death as compared to PrP<sup>C</sup> knockout (PrPKO) cells. Also, manganese-induced reactive oxygen species production, caspase-3 and caspase-9 activation, and DNA fragmentation were significantly lower in PrPC cells as compared to PrPKO cells (Choi et al., 2007), demonstrating that endogenous normal prion protein protects against metal-induced oxidative stress and neurotoxicity. Thus, our previous studies suggested that cellular prion protein may act as a metal sink, thereby preventing manganese from entering the cells and exerting its neurotoxic effect at the early stages of metal neurotoxicity.

Manganese has been shown to cross the blood-brain barrier via specific carriers such as transferrin and divalent metal transporter 1 and also by diffusion. The normal concentration of manganese in human adult tissues ranges from 3 to 20 μM, and the human blood manganese level is 7.2 μg/l, with a mean brain manganese level of 0.261 μg/g. Mn levels in the putamen, substantia nigra, and neuromelanin are 6.31, 0.34, and 58.5 ng/mg wet weight, respectively. Depending on the level of exposure, blood Mn concentrations can increase from 10- to 200-fold. Environmental exposures to manganese occur mainly by ingestion or inhalation. The U.S. Environmental Protection Agency reference limit for ambient manganese concentration is 0.05 μg/m<sup>3</sup>. Studies have shown that manganese levels range anywhere from 20,000 to 450,000 μg/m<sup>3</sup> in certain high exposure environments (Huang et al., 1989). In welding smoke, there might be more than 25,000 μg Mn per cubic meter (Wang et al., 1989). Normally, higher relative concentrations of manganese are required in cell

![FIG. 7. Manganese-induced PrP<sup>C</sup> upregulation in an infectious cell culture model of prion disease. (A) PK-resistant PrP<sup>Sc</sup> levels in RML-infected CAD5 cells. (B) Western blot analysis of PrP<sup>C</sup> at 12 h with 200 μM manganese treatment in both uninfected and RML-infected CAD5 cells. (C) Densitometry analysis of PrP<sup>C</sup> bands (17–37 kDa) in (A).](https://academic.oup.com/toxsci/article-abstract/115/2/535/1731367)
culture studies, and the concentrations used in our studies are consistent with other studies. In our previous dose response study in cell culture models of prion, 117 μM Mn was calculated as the EC₅₀ concentration (Choi et al., 2007), and therefore, we used 100 μM Mn for PrP⁰C cells. In order to achieve adequate intracellular concentrations of Mn in CAD5 cells and brain slices, we used 100 and 300 μM, respectively. The manganese concentrations used in our study are much lower than 0.6–1 mM concentration of Mn used in other cell types (Latchoumycandane et al., 2005; Marreilha dos Santos et al., 2008; Milatovic et al., 2009). Higher relative concentrations of test compounds are generally needed to elicit responses in cell cultures due to the acute nature of the treatment period in in vitro studies (hours to days) compared with chronic long-term studies in animal models (days to months). Importantly, the 100–300 μM manganese concentrations used in this study approximate the concentrations observed in the striatum of manganese exposed animals. Thus, the concentration of Mn used in the present study is consistent with the literature and relevant to Mn neurotoxicity (Marreilha dos Santos et al., 2008; Moreno et al., 2009).

While investigating the mechanisms of prion protein in metal neurotoxicity, we unexpectedly found that manganese treatment upregulates cellular prion levels. RT-PCR experiments revealed no increase in PrP⁰C mRNA levels. Additionally, studies with the transcriptional inhibitor actinomycin D showed mRNA stability is not altered during manganese treatment, suggesting that neither the transcription nor the mRNA stability of prion contributes to manganese-induced upregulation of PrP⁰C. Then, we examined whether upregulation of prion protein is due to impaired UPS and lysozyme activity, and the results indicated that both UPS and lysozyme protein degradative pathways are similar in manganese-treated and untreated cells. Alternatively, it is possible that manganese-induced increases in PrP⁰C levels could result from decreases in a key protease, calpain. However, studies have shown that calpain and other cytosolic proteases can be activated to increase the degradation of retro-translocated prion protein in the cytosol (Wang et al., 2005) and that calpain-dependent endoproteolytic cleavage of PrP⁰Sc modulates scrapie prion propagation (Yadavalli et al., 2004). A recent article by Sorgato and Bertoli (2009) suggests that a relationship between the prion protein and Ca²⁺ homeostasis exists, and the authors discuss the possibility that Ca²⁺ may be the factor behind the enigma of the pathophysiology of PrP⁰C (Sorgato and Bertoli, 2009). Since we did not see calpain-cleaved PrP⁰C degraded fragments in Western blots, it is unlikely that calpain played a major role in our study. As described in the following section, we believe that Mn-induced PrP⁰C upregulation may be the result of its binding to PrP⁰C octapeptide repeats.

This led us to examine whether manganese affects prion protein turnover rates in pulse-chase experiments. Manganese significantly altered the turnover rate of prion protein. The half-life of PrP greatly increased to 21 h in manganese-treated cells as compared to 13 h in untreated control cells, suggesting manganese treatment increases the stability of prion protein, which subsequently results in increased levels of prion protein during manganese exposure. We previously demonstrated that upregulation of prion protein does not occur in PrP⁰C cells during H₂O₂ or ER stress–induced apoptosis (Anantharam et al., 2008), suggesting that the altered prion protein turnover observed in this study probably results from Mn binding, which increases the stability of prion protein.

Although the physiological function of PrP⁰C remains to be elucidated, the metal-binding capacity of the protein is well recognized. The octapeptide repeat region of the protein structure has been established as key for the metal-binding role of PrP⁰C (Choi et al., 2006, 2007; Hooper et al., 2008; Todorova-Balvay et al., 2005). Numerous studies have shown that PrP⁰C has strong binding affinities to various divalent cations, including copper, manganese, and zinc (Choi et al., 2006, 2007; Hooper et al., 2008; Todorova-Balvay et al., 2005). One of the most significant differences seen with the absence of PrP⁰C in both mouse brain and cell cultures is the reduced basal content of crucial divalent cations, such as copper and manganese, strongly suggesting that PrP⁰C modulates metal homeostasis (Brown, 2009; Choi et al., 2007). Recent studies using recombinant PrP have shown that manganese can irreversibly replace copper bound to PrP, despite an apparent lower affinity, and this replacement causes conformational changes within the protein (Brazier et al., 2008; Brown et al., 2000). Recombinant PrP refolded in the presence of manganese was originally thought to bind up to four molecules of manganese at the octapeptide repeat domain, similar to copper (Brown, 2009). However, isothermal titration calorimetric studies demonstrated that PrP binds one molecule of manganese at each of two sites, with dissociation constants of 63 and 200 μM (Brazier et al., 2008). In comparison, divalent metal transporter 1, a known transporter of manganese across the plasma membrane, was shown in kinetic studies of manganese uptake to transport manganese in millimolar concentrations (Garrick et al., 2006). The biological consequence of copper replacement by manganese on the prion protein is yet to be established. Studies using circular dichroism and Raman optical activity indicate that upon PrP binding to manganese, the secondary structure becomes more organized, gaining greater α-helix and β-sheet content than copper-bound PrP (Brown, 2009). This suggests that manganese may promote misfolding and aggregation of prion protein that is a hallmark of prion diseases. Future investigation in a relevant animal model of chronic manganese neurotoxicity will help to address the role of manganese in prion protein misfolding and aggregation.

Our results show that manganese exposure increases PK resistance to prion protein in neuronal cells. Similarly, manganese exposure resulted in higher levels of PrP both in uninfected and in RML scrapie-infected CAD5 cells, suggesting that manganese treatment can affect PrP stability and expression even during the progression of prion disease. Brown
et al. have previously shown that primary rat astrocytes treated with manganese exhibit reduced proteolytic susceptibility, which they attributed to production of protease-resistant forms of PrP (Brown, 2009). Bocharova et al. (2005), however, have shown that manganese has little if any direct effect on recombinant PrP protease resistance or secondary structure. Interestingly, a study with yeast prion proteins also showed that yeast cells treated with manganese-supplemented media generated PK-resistant forms of PrP (Treiber et al., 2006). It appears that the interaction of metals with recombinant PrP protein in a cell-free system may be different from in vivo conditions. The biological relevance of manganese-induced prion protein upregulation is an important point to discuss. Studies from knockout mice as well as cattle have demonstrated that PrP(C) is an absolute requirement for propagation of prion disease (Fischer et al., 1996; Richt et al., 2007). Conversely, multiple copies of the PrP gene or PrP overexpressing transgenic mice show a shortened incubation time from infection to disease onset, resulting in exacerbation of the disease (Fischer et al., 1996). Since the normal cellular form of prion protein PrP(C) serves as a seed for conversion of the diseased form of prion protein, PrP(Sc), manganese-induced upregulation of PrP(C) could provide more substrate for spontaneous conversion of PrP(C) into PrP(Sc). The structural changes within the protein induced by manganese binding to the octapeptide repeats may explain the altered PK resistance and increased stability observed in this study. Whether manganese exposure alone can cause prion pathology remains to be investigated. However, the presence of higher levels of PK-resistant nonpathogenic prion protein can serve as a seed for PrP(C) conversion to pathogenic PrP(Sc), resulting in the acceleration of the prion disease progression. Together, this study points to the possibility of intracellular manganese impacting the availability of the PrP(C) substrate for conversion to PrP(Sc) and thereby contributing to the pathogenesis of prion diseases.

The metal-induced prion upregulation may have some implications not only for prion diseases but also for other neurodegenerative diseases. Recent studies suggest that prion-like pathogenic mechanisms may play a role in Parkinson’s, Alzheimer’s, and Huntington diseases (Miller, 2009; Olanow and Prusiner, 2009). Although other neurodegenerative diseases are not contagious like true prion diseases, the propagation of α-synuclein or amyloid aggregations closely resembles prion-like mechanisms, in which normal proteins self-aggregate and the misfolded protein can be transmitted to unaffected neurons. Recently, α-synuclein has been shown to be transported via endocytosis from affected neurons to neighboring neurons and to engrafted neuronal precursor cells in a transgenic model, resulting in the formation of Lewy body-like Parkinson’s disease (PD) pathology and apoptosis (Miller, 2009; Olanow and Prusiner, 2009). Recently, two independent studies revealed that the Lewy pathology appears to spread in grafted neurons in PD patient brains via prion-like propagation from the host tissues to tissue grafts (Kordower et al., 2008; Li et al., 2008). Interestingly, a recent study shows the polymorphism in prion octarepeats in Parkinsonism (Wang et al., 2009). Thus, our findings may provide further mechanistic insights into the pivotal role of metals in the pathological progression of prion disease and other neurodegenerative diseases.

In summary, our study indicates that manganese is an important factor that could interact with PrP(C) to stabilize the protein, leading to increased PrP(C) levels in neuronal cells. This in turn suggests that investigation of manganese interaction with PrP(C) could yield valuable insights into the functional role of PrP(C) in metal neurotoxicity, as well as the role of the metal in the pathogenesis of prion disease and other related proteinopathies, such as Alzheimer’s and Parkinson’s diseases. Future studies in animal models will elucidate the interaction of metals with prion protein and its role in disease processes.

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