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Short communication

Iron overload following manganese exposure in cultured neuronal, but not neuroglial cells

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Abstract

Our previous studies show that manganese (Mn) exposure inhibits aconitase, an enzyme regulating the proteins responsible for cellular iron (Fe) equilibrium. This study was performed to investigate whether Mn intoxication leads to an altered cellular Fe homeostasis in cultured neuronal or neuroglial cells as a result of disrupted Fe regulation. Our results reveal a significant increase in the expression of transferrin receptor (TfR) mRNAs and a corresponding increase in cellular ^{59}Fe net uptake by PC12 cells, but not astrocytes, following Mn exposure. These findings suggest that alteration by Mn of cellular Fe homeostasis may contribute to Mn-induced neuronal cytotoxicity.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Manganese; Iron; Transferrin receptor; Transferrin receptor mRNA; uptake; PC12 cell; Astrocyte

Intracellular iron homeostasis is post-translationally regulated by one of the iron regulatory proteins (IRPs), namely cytoplasmic aconitase (ACO1) or IRP-I. This protein contains a unique [4Fe–4S] cubane cluster in its active catalytic site, with one particularly labile Fe atom. ACO1 can selectively bind to mRNAs containing a stem-loop structure, also referred to as iron responsive elements (IRE) [4,11]. In iron-replete cells, ACO1 secures iron as part of its structure in the form of a [4Fe–4S] cluster. While this form of ACO1 binds poorly to mRNA, it can enzymatically catalyze the conversion of bound citrate to isocitrate. When cellular iron levels are insufficient, ACO1 assumes a [3Fe–4S] configuration, loses its cluster and enzymatic activity, and is transformed into an mRNA-binding protein. In the latter state, the enzyme binds with high affinity to IRE-containing mRNAs, inhibits translation of those mRNAs whose IRE's are 5′ (e.g., ferritin, succinic dehydrogenase, mitochondrial aconitase), and stimulates the expression of those whose IRE's are 3′ (e.g., transferrin receptor). The net result of this RNA–protein interaction is an increase in cellular Fe uptake and a decrease in Fe storage [4,9,11].

Our previous studies indicate that Mn exposure significantly alters cellular aconitase activity [19]. This may be primarily due to a close mimicry between Mn and Fe in their coordination chemistry, allowing Mn to compete with Fe and insert itself into the fourth, labile Fe binding site in the enzyme's active center. We postulate that such replacement, while suppressing ACO1's enzymatic catalytic function, would increase the protein's ability to bind to mRNAs encoding transferrin receptor (TfR), which in concert with a down-regulation of Fe storage may promote cellular Fe overload. Consequently, the excess cellular Fe in the neurons and/or neuroglia may produce reactive oxygen species and induce lipid peroxidation. However, the question as to whether Mn exposure ultimately influences cellular Fe regulation in neurons or neuroglia has never been explored.

Thus, we sought to determine whether Mn exposure altered cellular Fe homeostasis by acting on the expression of TfR mRNA. To accomplish this, we examined the steady-state levels of TfR mRNA in cultured PC12 cells

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and a primary culture of murine astrocytes before and after Mn exposure. We further investigated the effect of Mn exposure on cellular Fe net uptake by these two types of cells.

PC12 cells, derived from murine adrenal pheochromocytoma cells featuring neuronal characteristics including the ability to synthesize and store the catecholamines [8], were purchased from American Type of Cell Culture (ATCC, Manassas, VA). The cells were plated in 24-well (4 cm^2/well) trays and incubated in RPMI 1640 medium (ATCC) with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% heat-inactivated horse serum, and 5% FBS. The medium was changed every 2–3 days. A primary culture of astrocytes was established according to the procedure described by Goodman et al., [7]. The cerebral cortices of newborn Sprague–Dawley rats (Hilltop, Cottdale, PA) were removed and minced with a fine scissors followed by incubation with trypsin. The dissociated cells were filtrated through a 75-μm sieve. After centrifuging at 300×*g* for 5 min, the cells were cultured in EMEM with 10% FBS, 0.54% glucose, 0.23% sodium bicarbonate, and 100 units/ml penicillin–streptomycin per ml at 37°C in a 5% CO_2/95% O_2 atmosphere, and the medium was changed every 3 days. Under the phase-contrast microscope, the phase-light glial cells with large, oval nuclei and many cytoplasmic processes formed a confluent monolayer and dominated the culture for at least 2 weeks.

To investigate the effect of Mn on the expression of TfR mRNA, PC12 cells or astrocytes ($0.5–1\times10^7$ cells per well) were exposed to 200 μM Mn (as $MnCl_2$) dissolved in culture medium at 37°C for 4 days. At the end of exposure, the cells were washed with PBS, and lyzed in RNA Zol B for Northern blot analysis of TfR mRNA. A detailed method for analysis of TfR mRNA has been previously described [20]. In brief, total RNA (20 μg) from the control and Mn-treated PC12 cells was electrophoresed on 1% formaldehyde–agarose gels, followed by hybridization with 1×10^7 cpm/ml of random-primed [^{32}P]-labeled TfR cDNA probe. The probe was 507 base pair fragment of 5′-end of a full length 3.4 kb rat transferrin receptor cDNA, subcloned to pTZ19U-RTR, which was a generous gift of Dr. Griswold at Washington State University [14] and has been used in our previous studies [20]. To quantitate TfR mRNA, the filters were hybridized with random-primed [^{32}P]-labeled β-actin RNA. The X-ray images of the Northern blots were scanned and analyzed using NIH Image 1.57 software package. The densities of TfR mRNA in control and Mn-treated groups were normalized by those of β-actin mRNA in each corresponding lane. For studies conducted on primary culture of astrocytes, total mRNA extractions were further purified by running through a mini-oligo (dT) cellulose column (5 Prime–3 Prime, Inc, Boulder, CO). The purified poly A mRNA fraction was then applied for assays of TfR mRNA according the aforementioned method.

The cellular Fe net uptake study was conducted on cultured cells (5×10^5 cells per well) by preincubation of the cells with various concentrations (50–200 μM) of Mn as $MnCl_2$ in normal growth medium for 3 days. At the time of the Fe uptake study, the growth medium was removed and the cells were rinsed rapidly three times with 1 ml of Hanks balanced solution. The Fe uptake study was initiated by incubating cells with 0.5 ml of uptake medium containing 2 μM $FeCl_3$, 0.05 μCi $^{59}FeCl_3$ and 0.67 μM transferrin (1/3 of Fe^{3+} concentration) in serum-free MEM. The cultures were then returned to the incubator. At each time point, the uptake was terminated by rapid aspiration of the medium, followed by thorough washes for three times using 1 ml of ice-cold washing buffer containing 0.29 M mannitol and 0.5 mM $CaCl_2$ in 10 mM Tris, pH 7.4 adjusted by HNO_3. The cells were then scraped off using a rubber policeman in 200 μl of washing buffer and transferred to centrifuge tubes. Following centrifugation at 13,000×*g* for 5 min, the pellet was rinsed with 200 μl of washing buffer three times and finally resuspended in 400 μl of washing buffer. The cells were then disrupted by sonication (VWR Sonifer Model 250) at a setting of 20% Output and 3.5 Control for 20 pulses. An aliquot (100 μl) of cell homogenate was used to count ^{59}Fe radioactivity using a Packard model Cobra-II gamma counter, and the other aliquot (100 μl) of preparation used for assay of protein concentrations.

The method of Bradford [5], using bovine serum albumin as the reference, was used for all protein determinations.

Statistical analyses of the differences in optical densities between the two groups were performed using Student's *t*-test. The time courses of cellular Fe uptake as affected by Mn exposure were analyzed by two-way analysis of variance (ANOVA). The differences between the two groups were considered significant if *P*-values were equal to or less than 0.05.

Following exposure of PC12 cells to Mn at 200 μM for 4 days, the expression of TfR mRNA was visibly increased on an autoradiograph of a Northern blot (Fig. 1A). Quantitative analysis of the optical abundance of the bands corresponding to TfR mRNA, which was normalized by that of β-actin mRNA in the same lane, revealed that Mn treatment elevated the level of TfR mRNA in PC12 cells by 30% of the control levels ($P<0.005$) (Table 1). Since Mn is known to alter aconitase activity [19], the current observation appears to be consistent with the hypothesis that Mn, by acting on the iron regulatory protein, may stabilize the mRNAs encoding TfR, although how closely this in vitro observation resembles the toxic effect of Mn in vivo remains to be explored.

Mn-stimulated expression of TfR mRNA has been previously demonstrated in choroid plexus epithelial cells, which may account for an enhanced distribution of Mn to the CNS [20,21]. In contrast, exposure of astrocytes to Mn did not show any significant changes in the expression of TfR mRNA (Fig. 1B, Table 1). Moreover, the relative

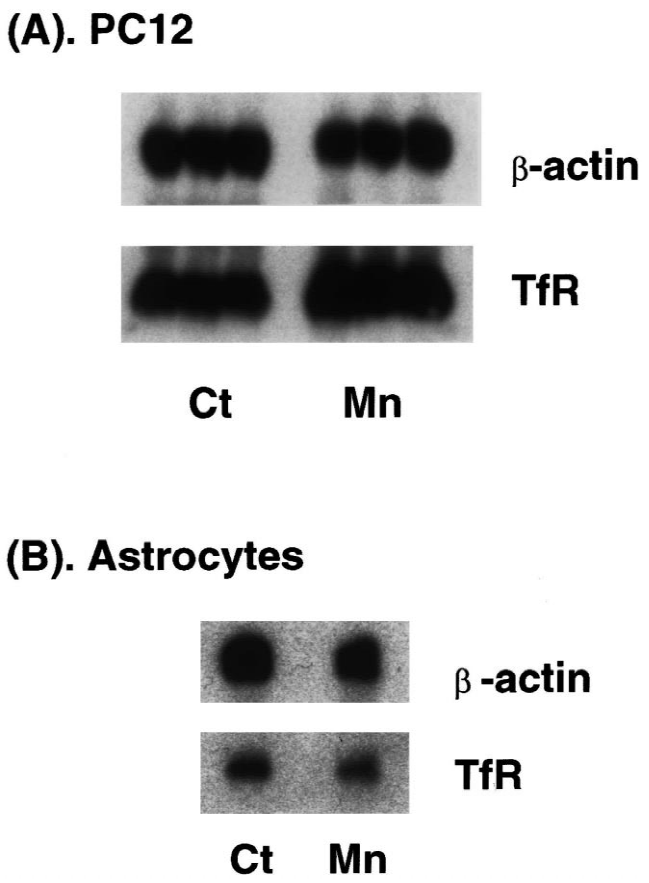

Fig. 1. Effect of Mn on the expression of TfR mRNA in cultured PC12 cells or astrocytes. The cells were incubated with 200 μM Mn (as $MnCl_2$) dissolved in culture medium for 4 days. (A) Northern blot of total RNAs from PC12 cells. (B) Northern blot of purified mRNAs from astrocytes. The optical density of TfR mRNA bands was normalized by the density of β-actin mRNA. Ct: control cells; Mn: Mn-treated cells.

optical density of TfR mRNA from control, untreated cells was much weaker in astrocytes than in PC12 cells (Fig. 1A vs. 1B) or in plexus epithelial cells [20]. While a total RNA extraction was sufficient for detection of TfR mRNA in PC12 cells or plexus cells, the same amounts of total RNA did not yield detectable autoradiographic signals for astrocytic TfR mRNA, unless a purified mRNA fraction was employed.

Exposure of PC12 cells to Mn caused a significant increase in cellular net uptake of ^{59}Fe (Fig. 2A). Two-way ANOVA revealed that cellular ^{59}Fe uptake was significantly associated with Mn concentration, exposure time, and concentration-by-time interaction (P values: for dose, $P<0.0001$; time, $P<0.0001$; dose×time, $P<0.05$). The cellular uptake of ^{59}Fe by PC12 cells was characterized by (1) a rapid uptake in the early stage followed by a slow decline of cellular Fe in both Mn-treated and control groups, and (2) a much higher ^{59}Fe level in Mn-treated cells than in the controls in the later phase. The maximum uptake of ^{59}Fe by both groups of cells occurred at 20 min following addition of ^{59}Fe into the culture medium, a time when Mn treatment elevated the cellular Fe load by 22% of control values. By 60 min, cellular Fe concentrations in both groups declined, suggesting redistribution and subsequent release of unbound Fe into the culture medium. The maximum percentage of Mn-facilitated cellular overload of Fe took place at 120 min, when an increase by 110% of control values was observed. The promotive effect of Mn on Fe uptake was Mn-concentration dependent within the range of 50–200 μM Mn in culture medium (Fig. 2B).

Table 1
Effect of manganese exposure on the expression of mRNAs of TfR in cultured PC12 cells and in a primary culture of rat astrocytes[a]

	Control	Mn-Treated	% Increase
PC12 cells	0.854±0.036	1.114±0.062	30.4 **
Astrocytes	0.423±0.179	0.478±0.202	13.0

[a] The optic density of bands for TfR mRNA was normalized to the abundance of β-actin mRNA in the same lane, and data represent the ratio of TfR/β-actin abundance. Means±S.D., $n=3$. **: $P<0.005$ as compared to the control.

When cultured astrocytes were treated with Mn at 50, 100, or 200 μM for 3 days, no significant difference in cellular net uptake of ^{59}Fe was observed between Mn-treated and control groups (only 200-μM experiments shown in Fig. 2C). Notably, the maximum net uptake of ^{59}Fe in normal, untreated astrocytes (27.3±3.9S.D. dpm/μg protein) was about 10 times less than that of control PC12 cells (266.4±10.8 dpm/μg protein).

Our observations raise a number of questions. First, what are the consequences of cellular overload of Fe following Mn exposure? Unlike other metals, excess intracellular Fe actively participates in generation of Fe-mediated reactive oxygen species (ROS), leading to neuronal cell death [10,18]. Thus, Mn, by elevating intracellular free Fe levels, may elicit oxidative stress and potentiate oxidative damage to neurons. Such a mechanism has been partly shown to occur in our recent in vivo study [20]. In Mn-exposed rats, the expression of mRNAs encoding glutamine synthetase, an enzyme which is highly sensitive to the oxidative stress [16,17], was increased as much as 34% of the control. This is presumably due to an aggrandized feedback-synthesis of glutamine synthetase as a result of Fe/ROS-mediated enzyme inhibition [20]. Based on these findings, we propose in Fig. 3 a mechanistic pathway of Mn cytotoxicity. Upon entering the cells, Mn may compete with Fe for [Fe–S] containing enzymes and alter iron regulatory proteins (e.g., ACO1). The latter impels an up-regulation of TfR for Fe uptake and down-regulation of ferritin for Fe storage, which together raise the intracellular free Fe. A direct consequence of increased free cellular Fe is the production of highly reactive hydroxyl free radicals, which thereby induce the oxidative stress.

Second, the results of this study demonstrate a differential sensitivity in cellular Fe net uptake between neuronal type PC12 cells and neuroglial astrocytes. Does this suggest a distinct role of neurons and neuroglia in Mn disposition in the CNS? Does the presence and/or the

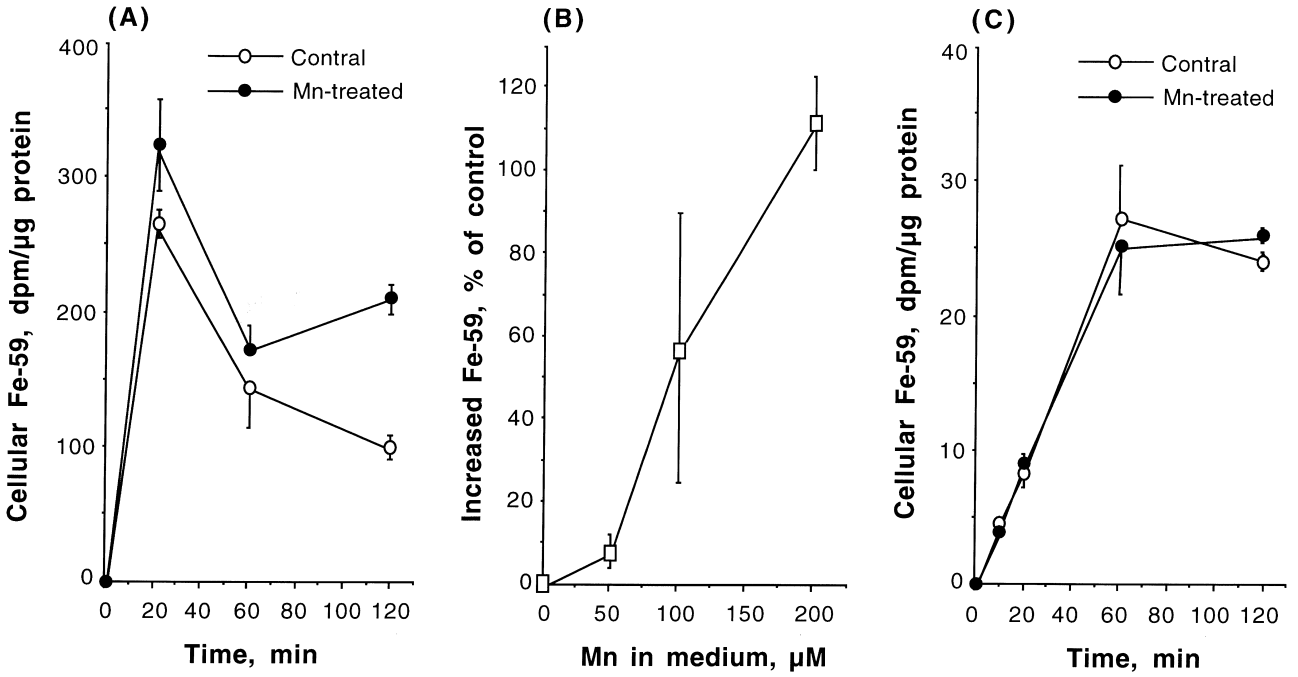

Fig. 2. Effect of Mn on cellular Fe net uptake in cultured PC12 cells or astrocytes. Cultured PC12 cells or astrocytes were pretreated with 200 μM Mn in culture medium for 3 days. (A). Time course study of PC12 cells. ^{59}Fe as $FeCl_3$ (2 μM) was added into the culture medium at time '0'. At the indicated times, cells were washed, homogenized, and counted for radioactivity. There was an overall statistically significant difference in cellular ^{59}Fe net uptake between the control and Mn-treated groups by two-way ANOVA ($P<0.001$). (B). Dose response study of PC12 cells. Cells were pretreated with 50–200 μM Mn in culture medium for 3 days, followed by incubation with 1.5 μM ^{59}Fe for 2 h. (C). Time course study of astrocytes. All data represent Mean±S.D.

degree to which TfR is expressed in brain cells contribute to cell type-selective cytotoxicity induced by Mn? The presence of TfR in astrocytes has been a subject of dispute. Connor and Menzies [6] have shown in their immunohistochemistry studies that TfR is expressed in neurons and to a less extent in neuroglia. Moos [12], on the other hand, reported a negative immunoreactivity of TfR in astrocytes, oligodendrocytes, or microglial cells. Our studies by Northern blot of TfR mRNA show that astrocytes do express TfR mRNA; but in a capability much weaker than PC12 cells or choroidal epithelial cells. The rather low base-level of TfR may partially explain the insensitivity of astrocytes to Fe cytotoxicity, although this cell type is known to accumulate as much as 80% of Mn in the brain [2,17]. Thus, we suspect that astrocytes in the brain may act as a metal depot for Mn ions. Whether or not there is a

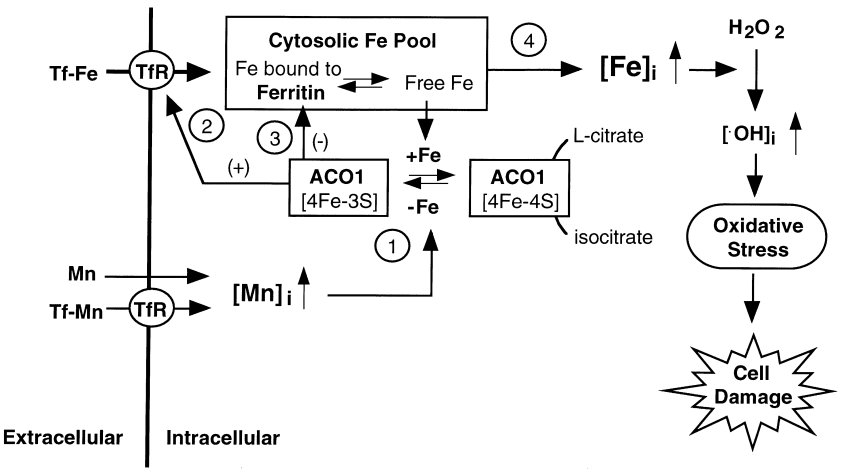

Fig. 3. Putative mechanism of Mn-induced cytotoxicity. Increased intracellular Mn alters iron regulatory protein (ACO1) in Step 1, leading to the up-regulation of TfR in Step 2 and down-regulation of Fe storage protein ferritin in Step 3. Increased TfR and decreased Fe storage elevate intracellular free Fe as shown in Step 4. The latter catalyzes the formation of highly reactive hydroxyl free radicals via Fenton reaction and provokes oxidative stress, resulting in ultimate cell damage.

threshold above which the capacity of astrocytes in sequestration of Mn is exceeded or saturated is unknown. However, a less disturbed cellular Fe homeostasis in astrocytes appears likely to protect them against Fe-mediated oxidative damage.

Finally, what is an exact toxic species of Mn in biological systems? The outer electron shell of Mn can donate up to 7 electrons, making it possible for Mn to assume 11 different oxidation states [3]. In living organisms, Mn has been found as Mn^{2+}, Mn^{3+}, and Mn^{4+} [1]. While it is quite reasonable to suggest that the effect on ACO1 activity was associated with the divalent oxidation species of Mn, our experimental design does not preclude the possibility that a Mn species of higher oxidation state, such as Mn^{3+}, is required for the induction of these effects. The ionic radius of Mn^{3+} is 65 ppm, which is similar to the ionic size to Fe^{3+} (65 ppm at the high spin state) in aconitase [13,15]. Thus, it is plausible that the higher oxidation state of Mn may optimally fit into the geometric space of ACO1, serving as the active species in this enzymatic alteration. This assumption, however, will have to await further experimentation.

In summary, these experimental results demonstrate a definite capacity of Mn to increase the level of TfR mRNA in PC12 cells, but not in astrocytes. The elevated TfR in neuronal cells may lead to the cellular overload of Fe and subsequently initiate Fe/ROS meditated cytotoxicity. The implications of these findings and the eventual mechanisms by which brain cells express differential sensitivity to Mn should be of interest to researchers, clinicians, and industrial hygienists.

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