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Original Contribution

MODULATION OF OXIDATIVE EVENTS BY MULTIVALENT MANGANESE COMPLEXES IN BRAIN TISSUE

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Abstract—Manganese toxicity can evoke neuropsychiatric and neuromotor symptoms, which have frequently been attributed to profound oxidative stress in the dopaminergic system. However, the characterization of manganese as a pro-oxidant remains controversial because antioxidant properties also have been associated with this metal. The current study was designed to address these disparate findings concerning the oxidative properties of manganese. The apparent ability of manganese in its divalent form to promote formation of reactive oxygen species (ROS) within a cortical mitochondrial-synaptosomal (P2) fraction was completely abolished by the addition of one five hundredth of its molarity of desferroxamine (DFO), a trivalent metal chelator. This large ratio and the high specificity of DFO for trivalent metal ions discounted the possibility of inhibition of ROS generation by direct sequestration of divalent manganese, and implied the trace presence of a trivalent metal. Further analysis suggested that this trace metal was manganic rather than ferric ion. Ferric ion was able to dampen the reactive oxygen species-generating capacity of manganous chloride, whereas manganic ion markedly promoted this property attributed to manganous ion. Such findings of the potent effects of trace amounts of trivalent cations upon Mn^{2+}-related free radical generation offer resolution of earlier disparate findings concerning the oxidative character of manganese. © 2001 Elsevier Science Inc.

Keywords—Manganese, Reactive oxygen species, Oxidative stress, Pro-oxidant, Antioxidant, Neurotoxicity, Transition metals, Free radicals

INTRODUCTION

Chronic exposure to manganese devastates the central nervous system with symptoms strikingly similar to schizophrenia and Parkinsonism [1,2]. Brain lesions, marked by neuronal degeneration [3], are focal within brain regions that are particularly active in oxidative metabolism and have a significant dopamine content, such as the rat hypothalamus or primate substantia nigra [4]. Due to their high consumption of oxygen, these regions tend to generate large quantities of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$). These oxidative species readily participate in single-electron transfers with "catalytic" transition metals to generate oxygen-derived free radicals, or highly reactive oxygen species (ROS). Although the cell normally relies on transition metals to modulate and transport oxygen, the presence of free transition metals that are not bound to proteins creates the potential danger for promotion of unwanted free radical reactions. Such an excess of transition metals may exert a pro-oxidant effect and initiate neuronal degeneration.

The pro-oxidant effects of transition metals have been confirmed repeatedly in vitro and in vivo studies [5–7]. A small increase in levels of free iron within cells can enhance lipid peroxidation and dopamine auto-oxidation [8–10]. Similarly, in its oxidation from the univalent to the divalent form, copper reacts with lipid hydroperoxides to generate diene conjugates, and eventually cytotoxic alkoxyl, peroxyl, and lipid radicals [11]. The oxidation of iron, copper, and other transition metals is thought to catalyze the homolytic fission of hydrogen peroxide to hydroxyl radical and hydroxyl ion in the Fenton reaction [12].

The potential involvement of oxidative damage in manganese neurotoxicity suggests that manganese may

also catalyze the Fenton reaction. In spite of extensive research efforts, the evidence for such speculations of manganese pro-oxidant activity, based primarily on use of the divalent salt manganese chloride ($MnCl_2$), has been conflicting. Manganese has been implicated in enhancing dopamine auto-oxidation and oxidative nigral [10,13–15], and in inflicting damage similar to that of other mitochondrial toxins such as carbon monoxide and cyanide [16]. In evaluations of the oxidative character of manganese, several groups have attributed accelerated ROS formation to both the divalent and trivalent states of the metal [17,18]. However, other reports contend that divalent manganese ion scavenges the ROS species superoxide and hydroxyl radicals even when SOD activity is inhibited [19], and attenuates oxygen toxicity in the *Lactobacillus planetarium* and related bacteria deficient in the superoxide dismutase enzyme [20]. Additional anti-oxidant effects, including catalase-like disproportionation of H_2O_2 [21], inhibition of iron-induced lipid peroxidation and copper-dependent low-density lipoprotein conjugation [7] have also been demonstrated using the same divalent salt $MnCl_2$. Conflicting findings such as these have complicated the characterization of manganese as either a pro-oxidant or an antioxidant.

The current study was designed to address these inconsistencies by focusing on the oxidative capacity of Mn^{2+} and Mn^{3+}, the two primary valence states of manganese in biological tissues. The findings indicate that manganese indeed may promote unwanted free radical reactions, however, the redox dynamics between its lower and higher valence states differ from that of other pro-oxidant metals. The key finding is that the trace presence of a trivalent metal ion is required for the pro-oxidant activity associated with excess Mn^{2+}. The catalytic effect of nanomolar concentrations of the trivalent ion on the oxidation of Mn^{2+} and redox cycling may account for earlier conflicting reports pertaining to the oxidative properties of manganese salts.

MATERIALS AND METHODS

Tissue preparation

Brains obtained from 2–3 month old male B6C351 strain mice were excised on ice, and the cerebral cortex was weighed and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 $\times$ g for 10 min. The resulting supernatant fraction was centrifuged at 31,500 $\times$ g for 10 min to yield the crude mitochondrial/synaptosomal pellet (P2). This was taken up in HEPES buffer to a concentration of 0.1 g-eq/ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10 at pH 7.4.

Assay for reactive oxygen species formation

Reactive oxygen species (ROS) were assayed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [22]. DCFH is capable of being oxidized to the fluorescent 2′,7′-dichloro-fluorescein by reactive oxygen species. Although the identity of the ultimate species responsible for the oxidation of DCFH is not known [23], the utility of this probe as a sensitive measure of ROS in biological systems, including isolated subcellular cerebral systems, has been documented [24].

P2 suspensions were diluted in 19 volumes of HEPES buffer. Test compounds were pipetted into 96 well cluster plates (Costar, Corning Inc., Corning, NY, USA) with four replicates per sample, and suspended in 100 μl of Tris-buffer per well. A volume of 100 μl, containing 5 μl of P2 fraction and 95 μl of 5 μM DCFH-DA, was added to each well for a final volume of 200 μl.

Given the light-sensitive nature of the probe, assays were carried out in a FL600 spectrofluorometer (Biotek Instruments, Inc., Winooski, VT, USA). The microplate reader allowed for fast light excitation and precise fluorescence capturing, thus minimizing photo-oxidation of the probe [25]. The incubation temperature was maintained at 37°C. Excitation and emission were set respectively at 485 nm and 530 nm. The fluorescence from each well was monitored, digitized, and stored on a computer using KC4 (Version 4.0) (Biotek) software. Parallel blanks with no DCFH-DA were included, and this value was subtracted from readings of samples to correct for autofluorescence of fractions. Additionally, parallel blanks with no P2 loaded with DCFH-DA served as an indicator of photo-oxidation of DCFH-DA. A DCF standard curve (1–500 nM) was used for conversion of fluorescence to pmol DCF formed/μg protein/2 h.

Protein determination

Protein content was assayed using the method of Bradford [26].

Statistical analysis

Statistical analysis was performed using ANOVA and Fisher's Least Significant Difference Test to assess the significance of differences between groups. The acceptance level of significance was $p < .01$ using a two-tailed distribution.

RESULTS

Differential promotion of ROS formation by divalent and trivalent manganese

The rate of generation of ROS in a cerebral cortical mitochondrial-synaptosomal fraction was enhanced by
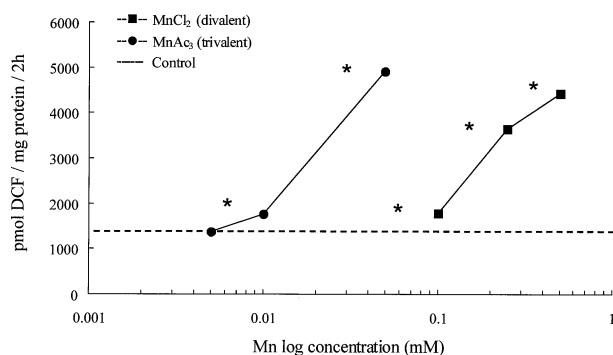
Fig. 1. Reactive oxygen species production as measured by DCF formation in P2 cortical fractions following in vitro exposure to $MnCl_2$ and $MnAc_3$. The DCF formation is plotted against the log concentration. Values are mean $\pm$ SE of three independent experiments realized in quadruplicate. *Value differs ($p < .01$) with that of the control. (Error bars are too small to visualize).

Fig. 3. Modulatory effects of trivalent iron and manganese salts on the formation of reactive oxygen species by $MnCl_2$. Values are mean $\pm$ SE of three independent experiments realized in quadruplicate. *Value of manganese-treated fractions differs ($p < .01$) with that of the control; +value of fractions treated additionally with either $MnAc_3$ or $FeCl_3$ differs ($p < .01$) with the corresponding value of fractions treated with $MnCl_2$ alone.

100 to 500 μM of $MnCl_2$ (Mn^{2+}), whereas manganic acetate (Mn^{3+}) affected a similar increase in ROS formation at an order of magnitude lower in concentration (0.5 to 5 μM) (Fig.1).

Manganous-promoted ROS formation quenched by chelator DFO

Previous studies in this laboratory have found that interactions between different metal species, specifically lead and aluminum, as well as the transition metals iron, copper, and chromium, accelerate ROS formation [27, 28]. To ascertain the contributions, if any, of iron to manganese-promoted ROS formation, desferroxamine (DFO), a widely used chelator of trivalent iron and aluminum, was incubated with each of the manganese salts (Fig. 2). This was intended to deplete traces of
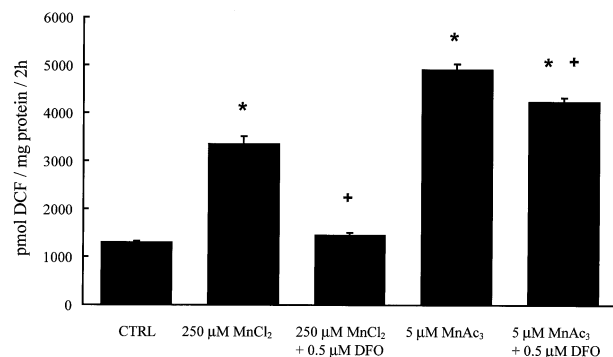

Fig. 2. Effect of DFO on manganese-generated ROS formation. P2 cortical fractions were incubated with or without one of the manganese salts. Values are mean $\pm$ SE of three independent experiments realized in quadruplicate. *Value of manganese-treated fractions differs ($p < .01$) with that of the control; +value of fractions treated additionally with DFO differs ($p < .01$) with the corresponding value of fractions treated with one of the manganese salts alone.
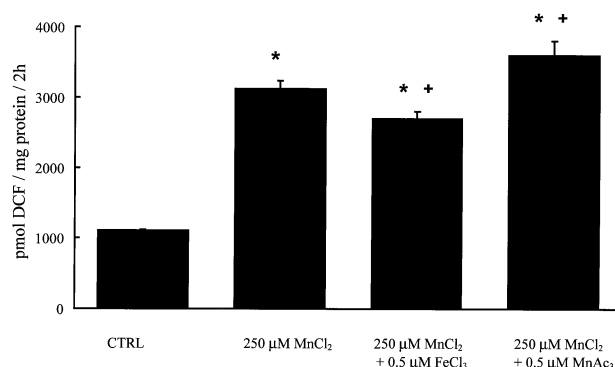
available ferric ions (Fe^{3+}) from the preparations. ROS formation by Mn^{3+} was attenuated by DFO at a molar ratio of ten to one. The rate of ROS formation was reduced yet the metal ion retained significant pro-oxidant activity. This minor reduction was attributed to sequestration of a significant amount of manganic ion.

In comparison, the effect of DFO on attenuation of ROS formation by Mn^{2+} was far greater than that of Mn^{3+}. Most remarkably, ROS levels were reduced below those of basal values in untreated subcellular fractions, even at a molar ratio of 500 to 1 of Mn^{2+} to DFO (Fig. 2). Because quenching occurred at such a large ratio between divalent manganese and DFO, the possibility of inhibition of ROS generation by sequestration of Mn^{2+} by DFO was discounted. The low concentrations of DFO required to quench ROS implied the trace presence of a trivalent metal.

Manganic but not ferric enhanced manganous-promoted ROS formation

Because DFO at nanomolar concentrations abolished Mn^{2+}-promoted ROS formation, the possible presence of very low concentrations of a trivalent metal ion was investigated to try to account for the pro-oxidant activity associated with Mn^{2+}. To simulate the effect of trivalent metal ions, which are intrinsically available in trace amounts in brain tissue, Fe^{3+} and Mn^{3+} salts were added at very low concentrations (500 nM–5 μM) to different samples incubated with $MnCl_2$ (Fig. 3). While additions of nanomolar concentrations of the ferric salt reduced Mn^{2+}-promoted ROS formation, additions at the same concentrations of the Mn^{3+} salt potentiated Mn^{2+}-promoted ROS formation. Additions of the Mn^{3+} salt alone
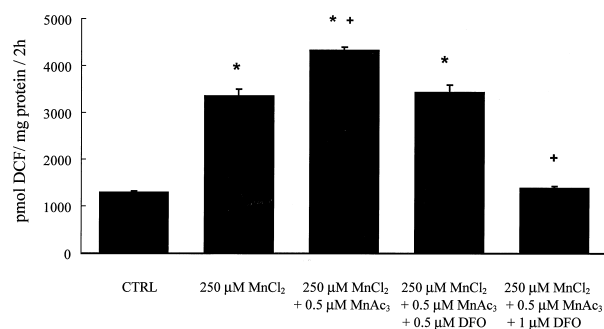
Fig. 4. Inhibition by DFO on manganese-based ROS generation. P2 cortical fractions were incubated with or without $MnAc_3$, and DFO. Values are mean ± SE of three independent experiments realized in quadruplicate. *Value of manganese-treated fractions differs ($p < .01$) with that of the control; +value of fractions treated additionally with $MnAc_3$ and/or DFO differs ($p < .01$) with the corresponding value of fractions treated with $MnCl_2$ alone.

at nanomolar concentrations did not promote ROS formation above basal levels (Fig. 1).

Chelation of manganic ion by DFO neutralized pro-oxidant activity of manganous ion

To inquire further whether Mn^{3+} may be the metal being chelated by DFO, promotion of ROS production in samples incubated with $MnCl_2$ was tested in the presence and absence of nanomolar and low micromolar concentrations of $MnAc_3$ (500 nM–2 μM) and DFO (500 nM–5 μM) (Fig. 4). A concentration of 500 nM DFO completely neutralized the effect of the added 500 nM Mn^{3+}, and reduced ROS production to levels similar to those found when Mn^{2+} was added alone. Furthermore, an excess of DFO (1 μM), intended to complex with the added 500 nM Mn^{3+} and any trace amounts of Mn^{3+} already pre-existing in solution, completely abolished the excess generation of ROS observed with addition of Mn^{2+} to basal levels.

DISCUSSION

The valence state of transition metals, such as iron and copper, has been established as a critical factor in the ability of the metal to catalyze ROS formation in the Fenton reaction, yet experimental use of Mn has been largely limited to that of the divalent salt. This may have been a critical oversight given that the redox cycling, which is necessary for the generation of ROS described by the Fenton reaction, involves at least two different valences. Indeed, studies of other valence states of manganese and their potential to promote oxidative stress have been sparse and conflicting [7,18,19,28].

The findings of this current study do not dispute either claims of Mn as a pro- or antioxidant. Rather such disparate characterizations of manganese can be reconciled by our finding that the pro-oxidant activity associated with divalent manganese depends on the trace presence of a trivalent metal ion, most probably manganic, Mn^{3+}. Although both valences of Mn have been associated with accelerated generation of ROS, the findings of this current study reveal a resistance to oxidation by Mn^{2+} that contrasts with the readiness of the lower valence states of iron and copper to become oxidized to higher valence levels.
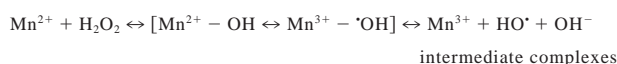
An apparent ability of manganese in the divalent state to promote the formation of ROS within a cortical mitochondrial-synaptosomal fraction was found. However, the addition of one five hundredth of its molarity of desferroxamine (DFO), a trivalent metal chelator, completely abolished the pro-oxidant activity of Mn^{2+}. The large molar ratio between additions of Mn^{2+} and DFO, and the high specificity of DFO for trivalent metal ions, negated the possibility of inhibition of ROS generation by sequestration of the divalent cation by the chelator. Moreover, the trace presence of a trivalent metal was implicated as a promoting factor in Mn^{2+}-related generation of ROS, given that DFO binds more tightly to trivalent ions than to divalent ones by at least 11 orders of magnitude [29]. Further analysis demonstrated that very low concentrations of Fe^{3+} ion were able to dampen the oxidative potential of Mn^{2+}, whereas small amounts of Mn^{3+} could markedly promote the ROS-generating capacity of Mn^{2+}. This suggested that the trace metal in manganous salts, which was complexed by DFO, was primarily Mn^{3+} rather than Fe^{3+}. Although DFO forms the most stable complex with Fe^{3+} [29], the ability of DFO to sequester trivalent metals such as Al^{3+} and Mn^{3+} has been utilized to study chelation therapy and metal coordination chemistry [30–33].

Mn^{3+} added alone at nanomolar concentrations was not sufficient to elevate basal levels of ROS formation, yet the same concentration range added to samples incubated with Mn^{2+} led to significant pro-oxidant activity. Moreover, nanomolar concentrations of DFO treatment of samples incubated with manganous and manganic salts returned the rate of ROS generation to basal levels. This implied an interaction between the divalent and trivalent states from which the capacity of Mn^{2+} to catalyze ROS generating reactions may be determined.

These data demonstrate the reluctance by manganese in the divalent form to undergo oxidation. Despite the feasibility of manganese catalyzing Fenton-like reactions [34], the low reduction potential of the aqueous divalent ion may not permit its interaction with H_2O_2 to produce $OH^{\bullet}$ at a measurable rate [29] under normal cellular concentrations and redox balance. However, the oxidation of Mn^{2+} may be facilitated by its interaction with

metal colloids and ligands, such as oxy-hydroxides, available in the biological environ [35]. Such catalysts can alter the electronegativity of the metal ion [36] and increase exponentially the rate constant of the oxidation reaction [37], thus overcoming the difficulty of the intrinsically low redox potential of the Mn^{2+} ion.

Mn^{3+} or intermediate complexes that are significantly stronger oxidizing agents may serve a similar catalytic role in the oxidation of Mn^{2+}. Given the redox potential between the two ions, Mn^{3+} or ionic intermediates could induce a shift in the electron density of the divalent ion to enhance its ability to oxidize or reduce. Effectively, an interaction between Mn^{2+} and ion catalysts could destabilize the divalent ion and change the overall redox status to one that is more favorable to the oxidation of the divalent ion and the acceleration of ROS generation.

$$Mn^{2+} + H_2O_2 \leftrightarrow [Mn^{2+} - OH \leftrightarrow Mn^{3+} - {}^{\cdot}OH] \leftrightarrow Mn^{3+} + HO^{\cdot} + OH^{-}$$
<div align="right">intermediate complexes</div>

Similarly, the dampening effect of the pro-oxidant activity observed with trace additions of Fe^{3+} suggest that this trivalent form of iron may also alter the redox status of Mn^{2+}. The symmetry of the d-electron configuration of manganous and ferric ions may confer stability to both the redox couples of manganese ($Mn^{2+} \longleftrightarrow Mn^{3+}$) and iron ($Fe^{2+} \longleftrightarrow Fe^{3+}$) by reinforcing the low redox potential of one another, thereby mitigating some of the pro-oxidant activity potentiated by Mn^{3+}.

The resistance of Mn^{2+} to oxidation differs with the readiness of the lower valence states of iron and copper to change to higher valence levels. For these latter transition metals, the lower valence state exhibits greater redox potential, higher reactivity, and greater readiness to catalyze free radical-generating reactions. The higher valence state Mn^{3+} showed significant redox potential, while the lower valence state Mn^{2+} was found to be a modest pro-oxidant only with the trace presence of another trivalent metal. Such a marked difference between the redox potential of the two valences contrast with other transition metals, suggesting that manganese may possess redox dynamics that rely heavily on other mediating factors before it undergoes free radical-generating reactions.

The known unusual ionic properties of its divalent and trivalent states of manganese validate the potential existence of atypical redox differentials. In contrast to other transition metals, manganese is most stable in aqueous, neutral pH solutions with the oxidation number of $+2$. Mn^{2+} is an S-state ion in the usual high-spin configuration with an approximately spherical distribution of the five unpaired $3d$-electrons about the nucleus. The half-filled $3d$ shell of Mn^{2+} imparts the ion with a stability analogous to that of the higher oxidation states of iron,

Fe^{3+}, and those of closed shell ions, such as those in the alkaline earth family (e.g., Mg, Ca) [38]. The stability of this electron configuration and the reluctance to lose a d-electron accounts for the poor reducing ability of Mn^{2+} [36]. Contrastly, Mn^{3+} has much higher transition probabilities compared to Mn^{2+} [38]. Its four unpaired electrons in the usual high-spin configuration are similar to those of the lower oxidation state of iron, Fe^{2+}, and render the ion with marked instability and high reduction and oxidation potential. Its high spin d^4, Mn^{3+} is inclined either to lose one electron in the antibonding e_g set of orbitals to maximize ligand field stabilization or to gain an electron to maximize electron exchange energy [36]. The hybrid properties of both closed shell and transition metal ions in Mn may manifest themselves as redox bioenergetics atypical of other transition metals.

The different properties of Mn^{2+} and Mn^{3+} and their interaction during oxidative events may be particularly relevant in the context of biological systems. Although the divalent state is the most stable form of manganese in aqueous solutions, the trivalent state is the dominant state of manganese in many biological tissues and in natural water sources [38]. In blood, free, and α2-macroglobulin-bound Mn^{2+} is oxidized to Mn^{3+}, possibly by ferroxidase I or ceruloplasmin, and is then bound to transferrin [39]. Within cells, manganese is found predominantly in mitochondria in the divalent form, where its uptake and export may be via a calcium carrier [38]. The high oxidative activity of some brain regions may facilitate oxidation of small amounts of divalent manganese to the trivalent state, thereby initiating Fenton cycling.

In summary, manganous ion has no intrinsic oxidant potential. However, Mn^{2+} may act in a pro- or antioxidant manner, depending on the trace presence of either Mn^{3+} or Fe^{3+} ion, respectively. Catalytic amounts of trivalent manganese may promote Fenton-type redox cycling, whereas ferric iron can attenuate this process. The potent effects of trace amounts of trivalent cations upon manganese-related free radical events may account for the discordant literature on this subject. Our findings can reconcile reports of inhibition of iron-based ROS production by manganese [40,7], and descriptions of the apparently intrinsic ROS-generating capacity of manganese in both divalent and trivalent forms [18]. The co-existence of both the divalent and trivalent forms of manganese in biological tissues underscores the potential metabolic significance of interactions of manganese in different valence states. This should serve as an impetus to reframe the design of studies on manganese intoxication—in terms of not only elements involved, but also of valence states.

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