

Manganese and Iron Interaction: a Mechanism of Manganese-Induced Parkinsonism

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ABSTRACT : Idiopathic Parkinson's disease (IPD) represents a common neurodegenerative disorder. While epidemiological studies have suggested a number of risk factors including age, gender, race, and inherited disorder, the cumulative evidence supports the view that environmental or occupational exposure to certain chemicals may contribute to the initiation and progress of Parkinsonism. More recently, clinical and laboratory investigations have led to the theory that dysregulation of iron, an essential metal to body function, may underlie IPD by initiating free radical reaction, diminishing the mitochondrial energy production, and provoking the oxidative cytotoxicity. The participation of iron in neuronal cell death is especially intriguing in that iron acquisition and regulation in brain are highly conservative and thus vulnerable to interference from other metals that bear the similar chemical reactivity. Manganese neurotoxicity, induced possibly by altering iron homeostasis, is such an example. In fact, the current interest in manganese neurotoxicology stems from two primary concerns: its clinical symptoms that resemble Parkinson's disease and its increased use as an antiknock agent to replace lead in gasoline. This article will commence with addressing the current understanding of iron-associated neurodegenerative damage. The major focus will then be devoted to the mechanism whereby manganese alters iron homeostasis in brain.

Introduction

Idiopathic Parkinson's disease (IPD) represents a common neurodegenerative disorder. An estimated 2% of the US population, aged 65 and older, develops IPD (Montgomery, 1995). This number will increase over the next several decades as the "baby-boomers" gradually step into this high-risk age group, concomitant with the increase in the average life-expectancy. While epidemiological studies have suggested the risk factors including age, race, gender, and inherited disorders in IPD, a close association between occupational exposure to certain chemicals and Parkinsonism has been documented (Cotzias, 1958; Ferraz *et al.*, 1988; Rybicki *et al.*, 1993; Tanner, 1989; Wang *et al.*, 1989). More recently, both clinical and laboratory investigations have led to the theory that the dysregulation of an essential metal iron may underlie IPD by initiating free radical reactions, diminishing the mitochondrial energy production, and provoking the oxidative cytotoxicity (Beard and Connor, 2003; Connor, 1997).

Exposure to manganese has long been known to

cause Parkinsonism (Barbeau, 1985; Chandra *et al.*, 1981; Gorell *et al.*, 1997; Mena *et al.*, 1967; 1970; Tepper, 1961). The neurodegenerative damage by manganese is usually irreversible and permanent. It is noteworthy that several Western countries, including the US, have now replaced lead in gasoline with the manganese-containing antiknock compound methylcyclopentadienyl Mn tricarbonyl (MMT). MMT has been reportedly associated with the increased health problems in heavily air polluted areas (Abbott, 1987; Cooper, 1984; Hakkinen *et al.*, 1983; Loranger and Zayed, 1995; Sierra *et al.*, 1995). The addition of MMT to the gasoline supply in the Western countries has raised a concern about the health risk related to a potential increase in the environmental levels of manganese.

This review deals with the current understanding of the role of iron and manganese in the etiology of neurodegenerative diseases. While there have been a substantial basic and clinical research on these two essential metals for the last half century, the mechanisms whereby iron and manganese induce neurodegeneration are still vague and even controversial. Thus, this article will stress mainly on the mechanistic aspect of iron and

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manganese neurotoxicity. For each metal, the primary utilities of the metal in human body will be briefly reviewed, followed by the discussion of the biochemical mechanisms underlying metal cytotoxicity. Inasmuch as the current view of the etiology of neurodegenerative diseases has arisen through the studies on the laboratory animals, the relevance of these studies to the environmental or occupational exposure will be particularly emphasized. Finally, future research needs in order to reveal the environmental ingredients that may contribute to the development of neurodegeneration are discussed. .

Iron Toxicity and Parkinson's Disease

Essential Functions of Iron and Its Homeostasis in Humans

Iron is one of the most abundant metals in the earth's crust, ranking only second to aluminum. Similar to other transition metals, iron exists in a variety of oxidation states from as low as Fe(-II) to as high as Fe(VI). The multiple oxidation states allow iron readily in taking part in oxidative-reduction reactions. Iron also forms the complexes through sulfur and oxygen ligands. Because of its subtle reactivity in chemistry, iron appears to be selected in molecular evolution to carry out a wide range of biological functions. A well-known function of iron in the body is to transport oxygen in hemoglobin and myoglobin. Iron is also required for the normal function of cytochromes, cytochrome oxidase, peroxidase, and catalase. Iron-sulfur proteins in mitochondrial respiration chain deliver the electrons generated from the tricarboxylic acid cycle (TCA cycle) to ADP molecules for energy production, thereby complying with the high demand of energy consumption of neuronal cells. In the brain, iron is required not only for DNA synthesis and mitochondrial respiration, but it also participates in the biosynthesis of neurotransmitters, axonal growth, and receptor-mediated postsynaptic signal transduction.

The total quantity of iron in the body averages 4 to 5 grams, about 65% of which is in the form of hemoglobin and 4% in the form of myoglobin. Another 15 to 30% is stored mainly in the reticuloendothelial system and liver parenchymal cells, primarily in the form of ferritin. The metabolism and systemic balance of iron in the body are precisely regulated by several macromolecules. Once iron enters the circulation from the small intestine, iron binds to a beta-globulin,

apotransferrin, to form transferrin. Transferrin carries iron in the blood and serves as the major vehicle for iron transport in the body. On arriving at the target cells, transferrin binds strongly with transferrin receptors (TfR) on the out surface of cell membrane. By endocytosis, transferrin carries iron to the cytoplasm or delivers iron directly to the mitochondria for heme synthesis. In the cytoplasm, the iron is released, and the free iron is either utilized in metabolic processes, or conjugated with a large molecular weight protein apoferritin (460,000 Dalton) to form ferritin. The latter acts as the storage for iron.

Under normal physiological conditions, the brain stringently regulates iron balance by three well coordinated systems: (a) the influx of iron into brain which is regulated by transferrin receptor-mediated transport at brain barriers; (b) the storage of iron in which the cellular sequestration is largely dependent upon availability of ferritin; and (c) the efflux of iron whose rate is controlled by bulk CSF flow to the blood circulation (Bradbury 1997; Connor and Benkovic, 1992; Jefferies *et al.*, 1984). At the brain barriers that separate the systemic circulation from the cerebral compartment (including the interstitial fluid and CSF), the iron-transferrin complex is taken up by endocytosis into cerebral capillary endothelia (and possibly into choroidal epithelia) where the molecules subsequently dissociate. Apotransferrin is then recycled to the blood compartment, whereas the released iron crosses the abluminal membrane of the barriers into the cerebral compartment by binding to brain transferrin derived discretely from oligodendrocytes and choroid plexus epithelia. The cerebral transferrin-bound iron thus becomes available for neurons expressing transferrin receptors (Beard and Connor, 2003). Hence, transferrin, transferrin receptor, and ferritin are quintessential to the regulation of iron in the CNS.

Cellular Iron Regulation

Cellular Fe homeostasis is tightly regulated by iron regulatory proteins (IRPs), one of which is cytoplasmic aconitase, or ACO1. Aconitase, whose structure contains a [4Fe-4S] cubane cluster in its active center, is an enzyme present in both mitochondria and cytoplasm (Henson and Cleland, 1967; Kennedy *et al.*, 1983). While cytosolic aconitase (ACO1) regulates intracellular Fe metabolism, the mitochondrial aconitase (ACO2) is responsible for the conversion of citrate to

isocitrate in the TCA cycle.

ACO1 controls iron balance by modulating the expression of mRNAs encoding the proteins involved in iron homeostasis. Through its [4Fe-4S] cluster, ACO1 binds to iron-responsive elements (IRE), which fold stereoscopically as a stem loop and locate in the 5' or 3' untranslated region of the mRNA sequence. Figure 1 illustrates the consequences of binding and unbinding of ACO1 to mRNA stem-loop structures of IRE. In Fe-replete cells, ACO1 secures Fe in the form of a [4Fe-4S] cluster. While this form of ACO1 poorly binds mRNA, it can enzymatically catalyze the conversion of bound citrate to isocitrate. When cellular Fe levels are insufficient, however, ACO1 assumes a [3Fe-4S] configuration, then loses its cluster as well as enzymatic activity, and is then transformed into an mRNA binding-protein. At this state, the enzyme binds with high affinity to IRE-containing mRNAs, inhibits translation of those whose IRE's are 5' (e.g., ferritin, SDH, mitochondrial aconitase), and stimulates the expression of those whose IRE's are 3' (e.g., Tf-receptors). The net result of this RNA : protein interaction is an increase in cellular Fe uptake. Therefore, cytosolic aconitase has been referred to as a protein with a "double life" (Henderson, 1996; Kennedy *et al.*, 1983).

The subcellular regulation of iron balance is so accurate that the process is highly sensitive to the subtle changes of each element involved. Thus, the disruption

in any of these regulatory processes would result in the profound consequences in cellular iron balance. It is noteworthy also that iron in brain is less mobilized than in other tissues or organs.

Iron Dysregulation in Parkinsonism

Abnormal iron homeostasis, both systemically and subcellularly, is believed to be associated with etiology of idiopathic Parkinson's disease (IPD) and chemical-induced Parkinsonism (Connor and Benkovic, 1992; Jenner *et al.*, 1992; Logroscino *et al.*, 1997; Youdim, *et al.*, 1993). In the substantia nigra of IPD patients, high levels of total iron, decreased ferritin, oxidative stress, and deficiency in mitochondrial functions, have been observed repeatedly (Dexter, *et al.*, 1991; Jenner *et al.*, 1992; Griffiths and Crossman, 1993; Loeffler *et al.*, 1995; Mann, 1994; Sofic *et al.*, 1991; Ye *et al.*, 1996; Youdim *et al.*, 1993). Recent studies by researchers at Columbia University have established that serum iron concentrations are significantly altered in IPD patients as compared to controls (Logroscino *et al.*, 1997). In addition, the serum ferritin, transferrin, total Fe-binding capacity (TIBC), and % Fe saturation are all reduced in IPD patients. These findings suggest a generalized "defect" in iron systemic metabolism in IPD.

Studies using animal models also reveal a general increase in the concentrations of iron in the substantia nigra following treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine

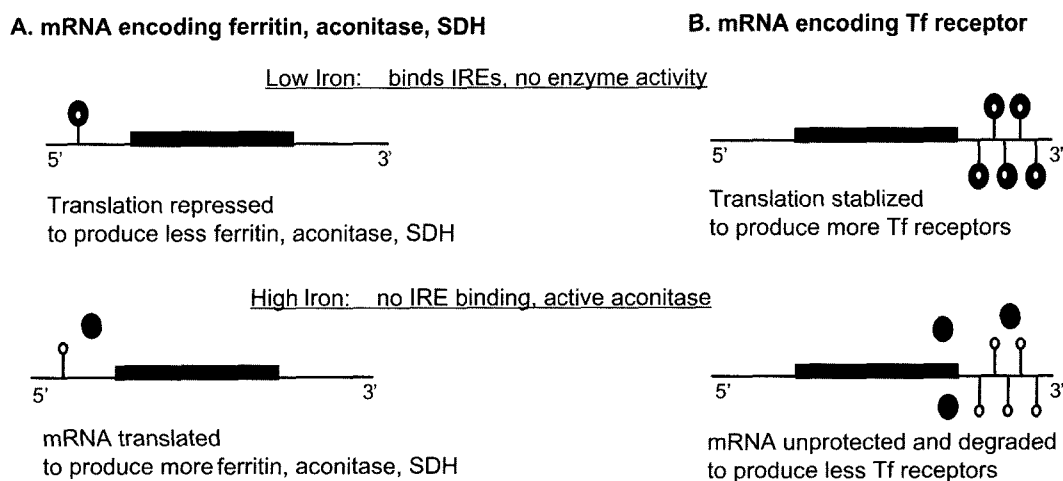
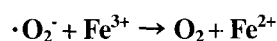


Fig. 1. Control of intracellular Fe homeostasis by IRP-I. Ovals represent IRP-I. Graph A on the left represents the mRNA encoding ferritin, etc. with one clustered IRE at 5' cap site. Graph B on the right represents the mRNA encoding Tf receptor with five clustered IRE's at 3' untranslated region.

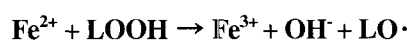
(6-OHDA), ibotenic acid infusion (to destroy GABAergic neurons), and direct intranigral iron infusion (He *et al.*, 1996; Mochizuki *et al.*, 1994; Sastry and Arendash, 1995; Sengstock *et al.*, 1992, 1994). It is still debatable as to whether iron overload in the substantia nigra is the cause or consequence of neuronal retrogression. For instance, the increased iron could directly result from the intervention of toxicants on iron regulation processes, which is followed by the subsequent neuronal cell death. It is also possible, however, that the increased iron may be a secondary event in the course of the cell injury to the cell death. In the latter case, the initiation of cell impairment may not be necessarily related to iron status. Despite these arguments, cellular iron overload in the substantia nigra may indeed represent the hallmark of the disease progress of the IPD. Accordingly, iron-mediated oxidative stress would lead to the further degeneration of nigrostriatal dopamine neurons in IPD patients or experimental animals.

Mechanism of Iron Cytotoxicity

As mentioned above, the multiple valent charges of iron molecule allow it to convert readily between oxidative-reductive state in biological matrices. One of the detrimental effects of iron's redox activity, however, is the involvement of Fe(II)/Fe(III) in the initiation of the cascade of free radical reactions. The free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital. Most free radicals are unstable and highly reactive. The radicals with an excess electron tend to donate electrons and in due course release Fe(III) from ferritin to produce free Fe(II) ions. The free Fe(II) ions, either released from their storage or presented in excess amounts, can catalyze the Fenton reaction, which generates highly toxic hydroxyl radical ($\cdot\text{OH}$) from superoxide radical ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2):



or, directly catalyze the decomposition of lipid hydroperoxides:



Free Fe(II) ions and cytotoxic $\cdot\text{OH}$ radical promote the oxidative reaction in polypeptides of proteins, nucleotides in DNA structure, or phospholipids in

membrane. The ensuing oxidation damage (i.e., oxidative stress) to cell membrane, enzyme activities, and nutrient production leads to the ultimate cell death of dopamine neurons in the substantia nigra of IPD. It is noteworthy that H_2O_2 species can readily cross the cell membrane; but its chemical reactivity is much lower than $\cdot\text{OH}$ species. The generation and presence of Fe(II), thus, is critical to the production of $\cdot\text{OH}$ and other reactive radical species.

Although it is still uncertain as to why iron accumulates in the specific area of IPD brain, the consequence of iron deposition in brain tissues has been associated with demyelination, altered synthesis of neurotransmitters, and impaired secondary messenger systems. Once iron in the brain, about 70-80% of iron is found in the myelin fraction, and histochemically it is localized in oligodendrocytes. The phenomenon of iron enrichment in oligodendrocytes has been repeatedly observed in mammalian brains across wide species (Beard and Connor, 2003; Dwork *et al.*, 1988; Erb *et al.*, 1996). One of the fundamental functions of oligodendrocytes is to produce a phospholipid myelin sheath around axons. The high demand of oligodendrocytes in iron acquisition and storage stems from the fact that iron, as a co-factor for cholesterol and lipid biosynthesis, is directly involved in myelin formation. The rich iron content, on the other hand, renders the oligodendrocytes particularly susceptible to oxidative injury. For example, in the animal model with experimental demyelination, both lipid and protein peroxides have been demonstrated (Beard and Connor, 2003; Smith *et al.*, 1999). In another animal model of experimental allergic encephalomyelitis, when the iron-chelating agent and some oxygen radical scavengers, such as superoxide dismutase and catalase, were applied to the animals, the treatment appeared to suppress the autoimmune demyelination (Bowern *et al.*, 1984; Hartung *et al.*, 1988). These studies suggest a direct engagement of iron in oxidative damage of myelin integrity in vivo. Loss of myelin not only disorients the neuronal firing, but it also leads to an increased oxidative stress in neurons. Nonetheless, in clinic, the relationship between the biomarkers, if any, of lipid peroxidation and oxidative stress and the brain region-specific damage has yet to be established in IPD patients.

Another factor that appears to impart iron toxicity is the metabolism of neurotransmitters. From the cumulative clinical documentation, the loss of striatal

dopamine is doubtlessly the primary cause of the IPD. There is a consistent minimum loss of 75% of the nigrostriatal dopamine neurons in IPD brains (Lloyd, 1977). In addition to the excitatory dopamine, the substantia nigra and globus pallidus (two iron-rich brain areas) receive an abundant innervation from the neostriatum, a considerable amount of whose neurons are GABAergic with an inhibitory neurotransmitter of GABA (γ -aminobutyric acid). Postmortem of IPD brains unveils that [^3H]GABA-binding in the substantia nigra of IPD patients is significantly decreased (Lloyd, 1977; Rinne *et al.*, 1984). Furthermore, a significant reduction of GABA concentration in the cerebrospinal fluid (CSF) of Parkinson's patients compared to the normal control subjects has been observed (De Jong *et al.*, 1984; Kuroda *et al.*, 1982). Some suggest that the declined GABAergic activity may be compensatory to the loss of dopamine in the same brain region (Lloyd, 1977). Others postulate a role of iron in GABAergic activity (Beard and Connor, 2003; Gabrielsson *et al.*, 1986; Palmeira *et al.*, 1993; Sohn and Yoon, 1998). A recent report indicates that GABAergic neurons appear to be more vulnerable to the iron toxicity than other type of neurons (Sohn and Yoon, 1998). Several lines of evidence suggest that iron cytotoxicity may affect both uptake and release of GABA by GABAergic neurons: (1) In rat brain synaptosome preparation, iron-dependent lipid peroxidation induced a severe decrease in synaptosomal uptake of GABA (Gabrielsson *et al.*, 1986; Rafalowska *et al.*, 1989; Medvedev *et al.*, 1992). (2) Following Fe(II)-induced membrane lipid peroxidation, GABA release was markedly reduced in synaptosomal preparations (Palmeira *et al.*, 1993) as well as in cultured cells (Rego *et al.*, 1996). (3) When iron was intraventricularly injected into rat brain, GABA release appeared to be more severely damaged than GABA uptake by lipid peroxidation (Zhang *et al.*, 1989). Thus, the iron toxicity to the striatal GABA neurons appears to contribute, at least in part, to the IPD.

Among the other neuronal processes that may adversely respond to iron accumulation, the interaction between iron and protein phosphorylation appears to be important, particularly concerning the role of protein kinase C (PKC) in the progress of cell death. PKC represents a family of Ca^{2+} - and phospholipid-dependent protein kinases that catalyze the transfer of the γ -phosphate of ATP to phosphoacceptor serine or threonine residues of protein and peptide substrates. PKC is

activated as a consequence of receptor-dependent increases in intracellular [Ca^{2+}] and diacylglycerol (DAG). Activation leads to the translocation of the enzyme from the soluble to membrane-associated particulate component of the cell. Protein kinase C (PKC) plays a critical role in transduction of cellular signals, in regulation of membrane functions, and in control of cell proliferation (Zhao *et al.*, 1998). A number of metal ions are capable of activating this cytosolic kinase (Hedberg *et al.*, 1994; Latorra *et al.*, 1992). The study from this laboratory has demonstrated that toxic metal lead activates PKC and promotes its translocation from cytosol to membrane in cultured choroidal epithelial cells (Zhao *et al.*, 1998). Recent studies further establish that activation of PKC may facilitate the programmed cell death or apoptosis (Villalba *et al.*, 1998). Some experiments have shown that application of specific PKC inhibitors can effectively prevent against ischemia-associated neuronal degeneration, glutamate-promoted excitotoxicity, NMDA receptor-mediated neuronal death, and chemical-induced neuro-cytotoxicities (Bhatnagar *et al.*, 1999; Buchner *et al.*, 1999; Felipo *et al.*, 1993; Fernandez-Sanchez *et al.*, 1996; Hammer *et al.*, 1993; Noh *et al.*, 1999; Savolainen *et al.*, 1995). Presumably, PKC may catalyze the phosphorylation of proteins involved in synthesis of $\cdot\text{OH}$ radicals.

Iron can modulate the expression and activity of PKC. When the cells are overloaded with excess Fe(III)-transferrin, the expression of PKC protein molecules is upregulated (Alcantara *et al.*, 1994). Iron can also facilitate the oxidation of PKC subunits to activate directly the PKC pathway (Taher *et al.*, 1993). Thus, in addition to the oxidative stress via Fenton reaction, interaction of iron with PKC may provide another path leading to the cellular oxidative damage.

Interestingly, the iron metabolism per se is partly regulated by cellular PKC. As mentioned above, cellular iron homeostasis is controlled largely by the action of the iron regulatory proteins (IRPs, Fig. 1). The activities of ACO1 are mutually exclusive and are modulated through the assembly/disassembly of its [4Fe-4S] cluster. PKC can catalyze the phosphorylation of [Fe-S] cluster in ACO1, thereby regulating its availability being reversibly as an IRE-binding protein or cytosolic aconitase (Brown *et al.*, 1998; Eisenstein *et al.*, 1993; Schalinske *et al.*, 1997). Apparently, modulation of PKC activity by other chemicals could subsequently

affect cellular iron balance.

Mn Toxicity and Parkinsonism

Essential Functions of Manganese in Human Body

Manganese exists abundantly in a number of physical and chemical forms in the earth's crust, water, and atmosphere's particulate matter. Manganese compounds including its mixture with iron are widely used in the manufacture of steel, in the production of batteries, in dietary supplements, and as an ingredient in some ceramics, pesticides, and fertilizers.

Because its outer electron shell can donate up to 7 electrons, manganese can assume 11 different oxidation states. Of environmental importance are Mn(II), Mn(IV), Mn(VII). In living organisms, manganese has been found as Mn(II), Mn(III), and Mn(IV). The ability of manganese to assume valence states ranging all the way from 3- to 7+ in combination with other elements is extremely important, emphasizing the potential for manganese to act either as a prooxidant or antioxidant in biological matrices.

Plants and animals cannot survive without the participation of manganese in their metabolic activities. In humans, food is the main source of manganese, and the usual daily intake ranges from 1 to 5 mg per day. As an essential nutrient, manganese actively participates in bone mineralization, protein and energy metabolism, metabolic regulation, cellular protection from damaging free radical species, and formation of glucosaminoglycans (Wedler, 1994). Manganese can activate numerous enzymes involved with either a catalytic or regulatory function such as transferase, decarboxylases, hydrolases, dehydrogenase, synthetases, and lyases. Mitochondrial superoxide-dismutase, pyruvate carboxylase, and liver arginase are known manganese metalloenzymes. Manganese is also required for many neuronal activities. The regulation of brain manganese depends largely on the blood-brain barrier and blood-CSF barrier. A primary transport form of manganese in blood (in the divalent oxidation state) crosses the blood-brain barrier via specific carriers at a rate far slower than in other tissues. The readers are alluded to a review article by Aschner *et al.* (1999).

Manganese-Induced Parkinsonism

Among the toxic metals, the relationship between

manganese intoxication and Parkinsonism has long been recognized (Barbeau, 1985; Chandra *et al.*, 1981; Gorell *et al.*, 1997; Mena *et al.*, 1967, 1970; Tepper, 1961). Health risks of exposure to Mn have been associated with organic Mn-containing pesticides, such as Mn ethylene-bis-dithiocarbamate (MANEB) (Ferraz *et al.*, 1988), and inorganic Mn dust or vapor among steel manufacturing workers or welders (Chandra *et al.*, 1981; Roels *et al.*, 1987; Wang *et al.*, 1989). The latter appears to be a more common source of exposure in developing countries including China (Wang *et al.*, 1998). A recent collaborative study between this laboratory with Dr. Dixin Wang at Beijing Institute of Labor Hygiene and Occupational Diseases has demonstrated a significant occurrence of Parkinson's syndrome among 3,200 welders employed in various professions in Beijing area. The manganese concentrations in blood or urine collected from chronically-manganese poisoned welders were significantly associated with aerial manganese levels in the breathing zone of the work sites (Wang *et al.*, 1998b). The other common source of manganese is found in the street drug called "Bazooka". It is cocaine-based drug contaminated with manganese-carbonate from free-base preparation methods (Ensing, 1985).

Recently, several countries, including the US, have replaced lead in gasoline with the manganese-containing antiknock compound methylcyclopentadienyl manganese tricarbonyl (MMT). MMT is a yellow volatile liquid with herbal odor that decomposes rapidly under the sun. In the US, MMT is produced by the Ethyl Corporation and marketed as HiTec3000 or AK-33X, which contains 24.4-25.2% manganese. MMT was initially introduced to gasoline as a supplement to tetraethyl lead in 1958. It was phased out in the US in the early 1970's, while Canada and several other European countries have been continuing using MMT as a gasoline additive up to this date (Frumkin and Solomon, 1997). In 1995, following a court ruling in favor of Ethyl Corp in Washington, D.C., MMT was reintroduced into the US market.

Combustion of MMT in the automobile produces manganese oxides, mostly in the form of Mn₃O₄ with less amounts of Mn₂O₃. The widespread use of MMT is expected to increase ambient manganese levels especially in the urban areas with high traffic. Some studies indicate that MMT uses may increase environmental Mn levels in other media as well, such as soil,

plants, and birds (Frumkin and Solomon, 1997). A more important fact, however, is the observation that MMT use causes increased health problems in heavily air polluted areas (Abbott, 1987; Cooper, 1984; Hakkinen *et al.*, 1983; Loranger and Zayed, 1995; Sierra *et al.*, 1995). One should be aware of that the introduction of organic lead to gasoline became one of the seminal environmental health catastrophes of the twentieth century. Keeping this in mind, a thorough assessment of economic, social, and health impact of manganese utilization is well warranted and timely.

Manganese-induced neurologic lesions are located in the globus pallidus and striatum of the basal ganglia. The pallidus and striatum display a marked decrease in myelinated nerve fibers, accompanied by depletion of striatal dopamine (Bonilla, 1980; Eriksson *et al.*, 1987; Ingersoll *et al.*, 1995; Mena *et al.*, 1967, 1970; Neff *et al.*, 1969; Yamada *et al.*, 1986). While the guideline to distinguish Mn neurotoxicity from IPD is still obscure, it is generally accepted that manganese specifically affects the striatum and pallidus, whereas IPD preferentially affects dopaminergic neurons of the substantia nigra (Inoue and Makita, 1996). Studies on monkeys suggest that following chronic exposure to manganese, neuroglial cells, i.e., astrocytes and oligodendrocytes, are the primary cellular target. These injured cells have been referred to as "bizarre glia" (Olanow *et al.*, 1996; Pentschew *et al.*, 1963).

Chemical and Biochemical Basis of Interactions Between Manganese and Iron

Chemically and biochemically, manganese shares numerous similarities with iron.

(a). Both metals are transition elements adjacent to each other in the Periodic Table. Manganese has the atomic number 25, while iron owns 26;

(b). Both metals have similar ionic radius. The electron configuration for manganese and for iron is -8-13-2 and -8-14-2, respectively;

(c). Both metals carry similar valent charges (2+ and 3+) in physiological conditions;

(d). In the body, both metals strongly bind transferrin (Tf) (Aschner and Aschner, 1990; Suarez and Eriksson, 1993; Ueda *et al.*, 1993);

(e). Intracellularly, both preferentially accumulate in mitochondria (Gavin *et al.*, 1990).

Because of these similarities, it is not surprising that manganese can interact directly with iron at the cellular

and subcellular levels, particularly on certain mitochondrial enzymes that require iron as a cofactor in their active catalytic center, such as aconitase, NADH-ubiquinone reductase (Complex I, Cpx-I), and succinate dehydrogenase (SDH).

Mechanism of Manganese-Induced Neurotoxicity

The mechanism of manganese-induced Parkinsonism has not yet been well defined at present. In view of the chemical similarities between manganese and iron, it is logical to postulate a mechanism by which manganese toxicity may interfere with certain critical iron-dependent CNS pathways or the iron metabolism itself.

Manganese Alters Systemic Iron Homeostasis.

Chronic Mn exposure in animals alters the systemic homeostasis of iron. Studies in this laboratory indicate that manganese appears to favor the influx of iron from the systemic circulation to the cerebral circulation (Zheng *et al.*, 1999a). In that study, groups of rats received intraperitoneal injections of MnCl₂ at the dose of 6 mg Mn/kg/day or equal volume of saline for 30 days. Rats exposed manganese resulted in a 32% decrease in plasma iron and no changes in plasma total iron binding capacity (TIBC). Much to the surprise, however, is that the iron concentration in the CSF of the same rats did not decline as did it in plasma. Instead, it increased by more than 3 fold of the control values. The ratio of CSF to plasma iron ($Fe_{CSF/plasma}$) was increased from 0.16 in control rats to 0.72 in the treated rats, reflecting an influx of iron from the systemic circulation to the CSF under the condition of manganese over exposure.

This pattern of plasma iron, while unexpected, is consistent with the observations in IPD patients whose circulating iron, ferritin, transferrin as well as total iron binding capacity are all significantly lower than those of control subjects (Logroscino *et al.*, 1997). The explanation for this overall deficiency in iron metabolism in IPD patients remain uncertain; however, manganese-induced diminished plasma iron may be attributed to manganese-enhanced intracellular distribution of iron. For example, Chua and Morgan (1984) observed that manganese supplementation in food led to an increased ⁵⁹Fe uptake by the brain, liver, and kidneys of rats. The authors concluded that manganese and iron interact during transfer from the plasma to the brain as well as other organs in a synergistic rather than competitive

manner. Seligman *et al.* (1988) also report that incubation of cultured human HL60 cells with a manganese-transferrin complex dramatically increased cellular uptake of ^{59}Fe . Furthermore, an in-vivo study showed that Mn intoxication caused an elevated Fe deposition in globus pallidus and substantia nigra pars reticulata of experimental monkeys (Olanow *et al.*, 1996).

That the chronic manganese exposure increases CSF concentration of iron suggests a unidirectional influx of iron into the cerebral compartment. Among the processes involved in brain regulation of iron, the entrance of iron to the cerebral compartment represents a critical step in monitoring cerebral iron homeostasis. It has been generally accepted that transferrin-bound iron gains access to brain via transferrin receptors on brain capillary endothelial cells (Jefferies *et al.*, 1986; Kissel *et al.*, 1998). Evidence also suggests that the choroid plexus, a major component of the blood-CSF barrier (Zheng, 1996, 2002), by producing transferrin for the CNS, participates in brain iron regulation (Dwork, 1995; Morris *et al.*, 1992). To further elucidate the role of brain barrier systems in the manganese-promoted influx of iron, we conducted a Northern blot study to quantitate mRNAs encoding transferrin receptor (TfR) in a well-established choroid plexus cell culture (Zheng *et al.*, 1998a, 1999b). When the choroidal epithelia were exposed to manganese (100 μM) for 14 hr, the expression of TfR mRNA was more abundant in manganese treated groups than in the controls (Zheng *et al.*, 1999a). Thus, it has become clear that the choroid plexus participates in the regulation of cerebral iron homeostasis, and this function can be modulated by excess manganese presented in the blood or accumulated in the choroid plexus. The degree to which the choroid plexus contributes to the overall brain iron regulation, as compared to the role of the blood-brain barrier, has yet to be established.

Manganese Alters Cellular Iron Homeostasis.

The rats chronically exposed to manganese display a substantial increase of manganese in brain tissues (Zheng *et al.*, 1998b). Is it possible that the increased manganese levels in brain tissue ultimately affect the cellular iron metabolism? To obtain the evidence so as to answer the question, an iron uptake study was performed in two major cell types of brain, namely, neuronal type PC12 cells and cultured astrocytes. Exposure of PC12 cells to manganese caused a

significant increase in cellular net uptake of ^{59}Fe . The cellular uptake was characterized by (1) a rapid uptake in the early stage followed by a slow decline of cellular iron in both manganese-treated and control groups, and (2) a much higher ^{59}Fe level in manganese-treated cells than in the controls in the later phase. The promotive effect of manganese on cellular iron uptake was manganese-concentration dependent within the range of 50-200 μM Mn in culture medium (Zheng and Zhao, 2001).

When cultured astrocytes were treated with manganese under the same condition, no significant difference in cellular net uptake of ^{59}Fe was observed between manganese-treated and control groups. The results demonstrate a differential sensitivity in cellular iron net uptake between neuronal type PC12 cells and neuroglial astrocytes. 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 iron cytotoxicity, although this cell type is known to accumulate as much as 80% of manganese in the brain. Thus, it seems likely that astrocytes in the brain may act as a metal depot for manganese ions (Zheng and Zhao, 2001).

These results provide the clue of the interactions between these two essential metals in the brain, when their supply surpasses their essentiality. Manganese interaction with iron appears to occur at multiple levels. At the systemic level, manganese may facilitate the iron uptake machinery at the brain barrier systems to allow more iron molecules to enter the CNS. Once in brain at the neuronal cellular level, manganese appears to promote cellular uptake of iron by various brain cell types, bringing about cellular iron overload by its own unique mechanism. The unbalanced brain iron status and the ensuing iron-initiated oxidative damage may initiate or promote the degenerative damage of neurons.

Manganese Interaction with ACO1, a Key Event Leading to the Dysregulation of Iron.

As discussed above, cytoplasmic aconitase (ACO1) functions to regulate cellular iron homeostasis through its unique [4Fe-4S] configuration. The presence of manganese in the surrounding medium may impair the reversible conversion of ACO1 as IRP or aconitase. In an in vitro study using purified aconitase, we found that incubation of the enzyme with Mn(II) reduced the

activity of aconitase by 81%. The inhibitory effect was dependent upon the Mn(II) concentration in the assay solution in the range of 10-50 μM with an ED_{50} about 64 μM . At 12.5 μM of Mn(II), there is a 24% inhibition in the enzyme activity ($p < 0.0073$). The aconitase activity in the reaction mixture is also dependent upon the iron concentration. Increased iron concentration in the reaction solution potently reversed the inhibitory effect of manganese on aconitase. The nature of this interaction is not completely understood at present; but it is conceivable that manganese may interact with iron in a competitive fashion.

In a recent study by this group, it is evident that Mn(III) species is more efficient than Mn(II) in inhibition of aconitase (Chen *et al.*, 2001). The enzyme activity was nearly entirely inhibited by Mn(III) at 10 μM in vitro. The ED_{50} for this action is about 5.4 μM of Mn(III). The study, thus, supports the view that Mn(III) may be more toxic than Mn(II).

Studies on the manganese and aconitase were also extended to animal model in vivo. Chronic exposure of rats to manganese (6 mg Mn/kg, i.p., for 30 days) led to a region-specific reduction in total aconitase (ACO1+ACO2) activities in brain tissue. In the striatum, reportedly a target for manganese neurotoxicity, manganese exposure caused a marked reduction (approximately 60% of controls) in aconitase activity, which approached, but did not attain statistical significance ($p < 0.0963$). The same treatment caused a 48.5% reduction of the enzyme in frontal cortex ($p < 0.01$), 20.6% in substantia nigra ($p < 0.139$), and 19.3% in hippocampus ($p < 0.323$) (Zheng *et al.*, 1998b).

Since the coordination chemistry of manganese closely resembles that of iron, it appears likely that manganese may insert itself into the fourth, highly labile iron binding site of both mitochondrial and cytosolic aconitase. The action, while suppressing the enzyme's catalytic function, may increase its binding

affinity to the mRNAs encoding major proteins in iron metabolism such as ferritin and transferrin receptor (Fig. 2).

Taken together, both these *in vitro* and *in vivo* data illustrate the sequential events following manganese exposure. The chronic manganese exposure results in the accumulation of manganese in the choroid plexus and brain tissues. The excess cellular manganese may stimulate the expression of transferrin receptor in the choroid plexus, and possibly at brain capillary endothelia (as well as in other peripheral organs). The overexpression of transferrin receptors at brain barriers and the ensuing facilitated iron transport from blood to the cerebral compartment would explain a compartmental shift of iron from the blood to the CSF. In the brain tissues, manganese, in the same manner as it does in the choroid plexus, may modulate aconitase activity and increase its binding affinity to the mRNAs containing IRE-stem loop. The net result of these interactions is an up-regulation of cellular iron uptake, which may provoke the iron-associated oxidative damage to neuronal cells.

Manganese Interferes Mitochondrial Energy Production.

Mitochondrion is not only rich in aconitase, but it is also the subcellular locale in which manganese and iron are preferentially sequestered (Gavin *et al.*, 1992; Beard and Connor, 2003; Romslo, 1980). As the interaction between manganese and aconitase becomes evident, the question as to whether manganese also acts on mitochondrial function was reasonably raised.

Mitochondria take up Mn(II) via the Ca(II) uniporter (Gavin *et al.*, 1992; Lehninger, 1972). The slow efflux of manganese by mitochondria accounts for the significant accumulation of this ion in the brain (Gavin *et al.*, 1990, 1992). Once sequestered in mitochondria, manganese inhibits Na^+ -dependent Ca^{2+} efflux, resulting in elevated levels of intramitochondrial Ca^{2+} . In a rat model, direct intrastriatal injection of Mn(II) into the

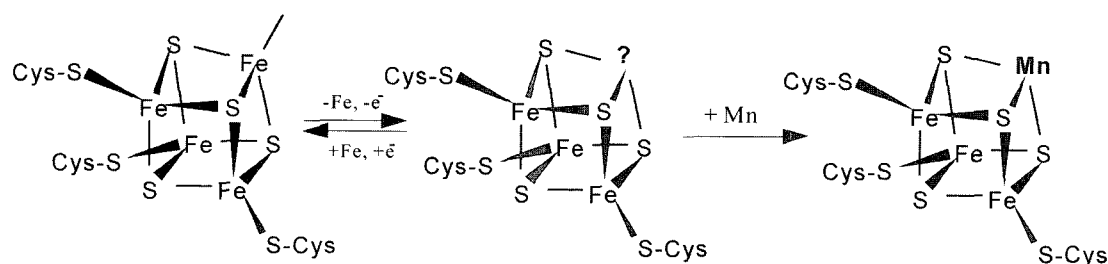


Fig. 2. Possible competition between Mn and Fe for the binding site in aconitase.

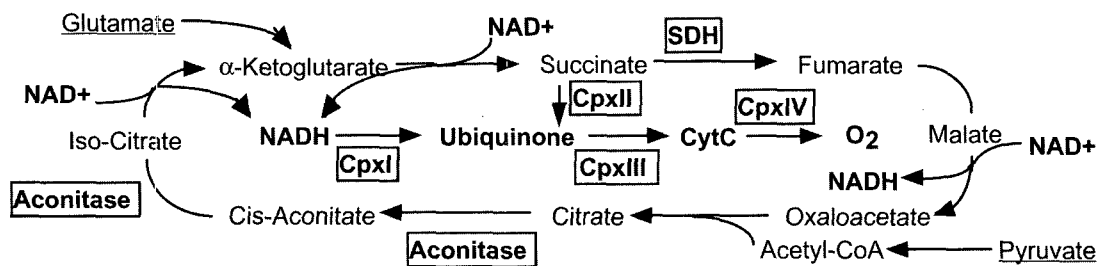


Fig. 3. Reactions catalyzed by aconitase, Cpx-I and SDH in the Krebs Cycle and electron transfer chain.

striatum reduces ATP level by 51% compared to the contralateral control hemisphere (Brouillet *et al.*, 1993). Manganese also decreases the rate of ADP-stimulated respiration, suggesting a direct effect on the electron transport system (Gavin *et al.*, 1990).

Aconitase, Cpx-I, and SDH, all of which require iron as a co-factor in their [4Fe-4S] active centers, catalyze the reactions involved in mitochondrial energy production (Fig. 3).

Mitochondrial aconitase (ACO2) has the same structure as the cytosolic ACO1. The enzyme is responsible for the conversion of citrate to isocitrate via the TCA cycle. While the reaction is not a rate limiting event in the ATP production, the alteration in enzyme activity by manganese, as discussed in the above sections, could contribute, to certain extent, the overall dysfunction of mitochondria.

NADH-ubiquinone reductase (Complex I, Cpx-I) is present in the inner membrane of mitochondria and catalyzes the transfer of two electrons from NADH to the small, lipid-soluble molecule ubiquinone, referred to as coenzyme Q. This reaction is the first step in the respiratory chain that delivers electrons to the energy storage molecule, ATP. The enzyme consists of 16 to 24 nonheme iron atoms in five to eight [Fe-S] centers (Albracht *et al.*, 1997). There is considerable evidence that Cpx-I activity is reduced in IPD substantia nigra, with levels of the enzyme reduced by 30-37% in IPD (Mann *et al.*, 1994; Mizuno *et al.*, 1995; Schapira *et al.*, 1990). Direct *in vivo* evidence of alteration of Cpx-I by manganese is still lacking. One report, however, showed that manganese reduced Cpx-I activity in cultured neuronal cells (Galvani *et al.*, 1995).

Recently, this group has performed the studies to demonstrate the inhibitory effect of manganese on mitochondrial Cpx-I. Using decylubiquinone (DB) as an electron acceptor, antimycin A to block Cpx-III, and

KCN to inhibit Cpx-IV, it was found that incubation of mitochondrial fractions with manganese significantly suppressed Cpx-I activity (Chen *et al.*, 2001). Presence of rotenone, a known specific inhibitor of Cpx-I, completely arrested the electron transfer from NADH to DB, providing further evidence that the reaction was Cpx-I specific. Mn(III) species appear to be more efficient than Mn(II) in inhibition of Cpx-I activity.

Succinate dehydrogenase, or succinate-ubiquinone reductase (SDH, complex-II), is a second enzyme involved in the mitochondrial electron transfer chain (Fig. 3). This enzyme has 8 [Fe-S] bonds in three centers. Human studies also suggest a possible decreased activity of this enzyme in IPD (Singer *et al.*, 1995). Animal studies in a chronic Mn exposure model revealed a 51% decrease in SDH activity in the whole brain homogenates (Seth *et al.*, 1977; Singh *et al.*, 1974). As a part of Mn-Fe interaction studies, this group also examined the effect of manganese on SDH or Cpx-II. The assay employed succinate as an electron donor and $\text{KFe}_3(\text{CN})_6$ as an electron acceptor. The enzyme activity was expressed as a function of the rate of reduction of $\text{KFe}_3(\text{CN})_6$ by SDH at 400 nm. Following incubation with manganese, a significant repression of mitochondrial SDH activity (23% of control) was observed.

The copy numbers of mtDNA have been shown to be sensitive to oxidative stress and have been used as an indicator for the functional integrity of mitochondria (Melov *et al.*, 1999; Tabrizi and Schapira, 1999). Initiation of mtDNA transcription is mainly regulated in the D-loop region, which is reportedly more susceptible to oxidative stress than other regions. The high level of oxidative stress in mitochondria, resulting from excessive iron accumulation, altered Complex-I activity, and arrested energy production after manganese exposure, would be expected to reduce the level of mtDNA. This

seems to be true based on the results of Chen's study (Chen *et al.*, 2001). Following treatment of PC12 cells with 100 μM of either Mn(II) or Mn(III) in culture media for 3 days, the densities of 16-kbp bands corresponding to the mtDNA were reduced by 24% and 16% in Mn(II) and Mn(III) treated groups, respectively (Chen *et al.*, 2001).

Thus, the above studies suggest that manganese, in addition to its detrimental action on iron regulation, may alter the certain key enzymes involved in mitochondrial energy production. One should notice that there is an interrelationship between mitochondrial energy status and cellular iron balance. Mitochondria contain a significant amount of intracellular iron. Approximately one-third of cellular iron content is heme, as cytochromes, one-third in Fe-S proteins, and one-third represents a less well-defined group of non-heme, non-Fe-S proteins (Flatmark and Tangeras, 1977). While many factors are known to impair and compete for mitochondrial iron uptake, the most important regulating factor is the mitochondrial energy state (Koller and Romslo, 1978). All factors known to influence the energy potential also affect iron uptake in a parallel and similar fashion, e.g., the uptake is inhibited by respiratory inhibitors and uncouplers, and it is stimulated following energization of the inner membrane (Romslo, 1980). The resulting unbalanced iron uptake will affect overall cellular homeostasis and promote oxidative damage via the Haber-Weiss reaction. In view of the inhibitory effect of manganese on energy production and the high accumulation of both metals in mitochondria, it is possible that damage to mitochondrial energy production by manganese would consequently influence mitochondrial iron uptake and thus intracellular iron homeostasis.

Summary and Perspectives

Occupational and environmental exposure to manganese continue to represent a realistic public health problem in both developed and developing countries. Increased utility of MMT as a replacement for lead in gasoline creates a new source of environmental exposure to manganese. It is, therefore, imperative that further attention be directed at molecular neurotoxicology of manganese. A need for a more complete understanding of manganese functions both in health and disease, and for a better defined role of manganese in

iron metabolism is well substantiated. The in-depth studies in this area should provide novel information on the potential public health risk associated with manganese exposure. It will also explore novel mechanism(s) of manganese-induced neurotoxicity from the angle of Mn-Fe interaction at both systemic and cellular levels. More importantly, the results of these studies will offer clues to the etiology of IPD and its associated abnormal iron and energy metabolism.

To achieve these goals, however, a number of outstanding questions remain to be resolved. First, one must understand what species of manganese in the biological matrices plays critical role in the induction of neurotoxicity, Mn(II) or Mn(III)? In our own studies with aconitase, Cpx-I, and Cpx-II, manganese was added to the buffers as the divalent salt, i.e., MnCl_2 . While it is quite reasonable to suggest that the effect on aconitase and/or Cpx-I activities was associated with the divalent species of manganese, the experimental design does not preclude the possibility that a manganese species of higher oxidation state, such as Mn(III), is required for the induction of these effects. The ionic radius of Mn(III) is 65 ppm, which is similar to the ionic size to Fe(III) (65 ppm at the high spin state) in aconitase (Nieboer and Fletcher, 1996; Sneed *et al.*, 1953). Thus, it is plausible that the higher oxidation state of manganese optimally fits into the geometric space of aconitase, serving as the active species in this enzymatic reaction. In the current literature, most of the studies on manganese toxicity have used Mn(II) as MnCl_2 rather than Mn(III). The obvious advantage of Mn(II) is its good water solubility, which allows effortless preparation in either in vivo or in vitro investigation, whereas almost all of the Mn(III) salt products on the market are water insoluble. The difficulty in obtaining soluble Mn(III) products makes the comparison between two valent manganese species nearly infeasible. Thus, a more intimate collaboration with physiochemists to develop a better way to study Mn(III) species in biological matrices is pressingly needed.

Second, in spite of the special affinity of manganese for mitochondria and its similar chemical properties to iron, there is a sound reason to postulate that manganese may act as an iron surrogate in certain iron-requiring enzymes. It is, therefore, imperative to design the physiochemical studies to determine whether manganese can indeed exchange with iron in proteins, and to understand how manganese interacts with tertiary

structure of proteins. The studies on binding properties (such as affinity constant, dissociation parameter, etc.) of manganese and iron to key enzymes associated with iron and energy regulation would add additional information to our knowledge of Mn-Fe neurotoxicity.

Third, manganese exposure, either in vivo or in vitro, promotes cellular overload of iron. It is still unclear, however, how exactly manganese interacts with cellular iron regulatory processes and what is the mechanism underlying this cellular iron overload. As discussed above, the binding of IRP-I to TfR mRNA leads to the expression of TfR, thereby increasing cellular iron uptake. The sequence encoding TfR mRNA, in particular IRE fragments, has been well-documented in literature. It is therefore possible to use molecular technique to elaborate whether manganese cytotoxicity influences the mRNA expression of iron regulatory proteins and how manganese exposure alters the binding activity of IPRs to TfR mRNA.

Finally, the current manganese investigation has largely focused on the issues ranging from disposition/toxicity study to the characterization of clinical symptoms. Much less has been done regarding the risk assessment of environmental/occupational exposure. One of the unsolved, pressing puzzles is the lack of reliable biomarker(s) for manganese-induced neurologic lesions in long-term, low-level exposure situation. Lack of such a diagnostic means renders it impossible to assess the human health risk and long-term social impact associated with potentially elevated manganese in environment. The biochemical interaction between manganese and iron, particularly the ensuing subtle changes of certain relevant proteins, provides the opportunity to identify and develop such a specific biomarker for manganese-induced neuronal damage. By learning the molecular mechanism of cytotoxicity, one will be able to find a better way for prediction and treatment of manganese-initiated neurodegenerative diseases.

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