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Redox dynamics of manganese as a mitochondrial life-death switch

Matthew Ryan Smith¹, Jolyn Fernandes¹, Young-Mi Go, and Dean P. Jones*

Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University, Atlanta, GA 30322, USA

Abstract

Sten Orrenius, M.D., Ph.D., pioneered many areas of cellular and molecular toxicology and made seminal contributions to our knowledge of oxidative stress and glutathione (GSH) metabolism, organellar functions and Ca^{+2} -dependent mechanisms of cell death, and mechanisms of apoptosis. On the occasion of his 80th birthday, we summarize current knowledge on redox biology of manganese (Mn) and its role in mechanisms of cell death. Mn is found in all organisms and has critical roles in cell survival and death mechanisms by regulating Mn-containing enzymes such as manganese superoxide dismutase (SOD2) or affecting expression and activity of caspases. Occupational exposures to Mn cause “manganism”, a Parkinson's disease-like condition of neurotoxicity, and experimental studies show that Mn exposure leads to accumulation of Mn in the brain, especially in mitochondria, and neuronal cell death occurs with features of an apoptotic mechanism. Interesting questions are why a ubiquitous metal that is essential for mitochondrial function would accumulate to excessive levels, cause increased H_2O_2 production and lead to cell death. Is this due to the interactions of Mn with other essential metals, such as iron, or with toxic metals, such as cadmium? Why is the Mn loading in the human brain so variable, and why is there such a narrow window between dietary adequacy and toxicity? Are non-neuronal tissues similarly vulnerable to insufficiency and excess, yet not characterized? We conclude that Mn is an important component of the redox interface between an organism and its environment and warrants detailed studies to understand the role of Mn as a mitochondrial life-death switch.

Keywords

Heavy metal; Hydrogen peroxide; MnSOD; Neurodegenerative disease; Nutritional metal; Redox state

1. Introduction

Sten Orrenius taught us to follow simple principles in science: pursue scientific enquiry by collectively engaging the scientific community, seeking out scientific questions which others agree are fundamental, locating knowledgeable authorities and learning from them, identifying and acquiring the most powerful experimental approaches, and engaging gifted

*Corresponding author. Department of Medicine, Pulmonary Division, Emory University, 205 Whitehead Biomedical Research Building, 615 Michael Street, Atlanta, GA 30322, USA., dpjones@emory.edu (D.P. Jones).

[†]These authors contributed equally to this work.

and dedicated experimentalists to rigorously apply the methods to advance knowledge. Simple, enjoyable, rewarding.

With this in mind, the present review reflects on the toxicology of manganese (Mn). Geologists tell us that Mn is abundant in the earth's crust. Chemists tell us that Mn is redox active. Nutritionists tell us that Mn is an essential nutrient and required for many enzymes. Redox biologists tell us that Mn is especially important to protect mitochondria from oxidative stress. Toxicologists tell us that excess Mn is neurotoxic, and exposure scientists tell us that occupational exposure is of greatest concern. And Professor Orrenius would quickly remind us that all of this is un-interesting—that what is interesting is that we do not know whether this redox-active metal impacts programmed cell death during development, that we do not know whether Mn is a variable impacting necrosis or apoptosis in disease mechanisms, especially in non-neuronal diseases, and that we do not know whether there is sufficient variability in dietary sources and/or absorption to create selective vulnerability for nutritional deficiency or environmental toxicity. In this article, we summarize available literature with the purpose to help identify the “interesting” questions about Mn, cell death mechanisms and implications for health and disease.

2. The effects of nutritional and heavy metals on cell death and human health

Apoptosis is a highly regulated form of cell death that is crucial to cellular development and elimination, thereby maintaining tissue homeostasis. While many variations in mechanisms are known and other mechanisms of cell death occur, key characteristics include the need to be tightly regulated because the loss of regulatory control and excessive cell death has been observed in a number of diseases such as cancer, autoimmune disease and neurodegenerative disorders [1]. Trace metals are an inherent component of all life forms, and a number of metals [cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn)] are essential nutrients in mammals. Despite this, excessive intake, exposure and/or inhalation has been demonstrated to lead to detrimental health effects [2], and considerable literature exists to describe impacts on cell death mechanisms. With continuing industrial, agricultural and technological applications of metals, concern exists about excess exposure, especially under conditions contributing to excessive cell death.

Mn was ranked 139th amongst 785 hazardous substances in the ‘ATSDR 2015 Substance Priority List’, based on the frequency of occurrence at the National Priorities List Site (NPL), toxicity and potential for human exposure [3]. Thus, from an environmental and toxicological standpoint, there is a recognized concern about Mn. However, from this perspective, there are 138 substances of greater concern, so why focus on Mn?

As outlined below, Mn is an essential nutrient and therefore all humans must have exposure to be healthy. But Mn is similar to iron (Fe) in many properties, especially in being redox-active, i.e., undergoing electron transfer reactions under biological conditions. Mn accumulates in neuronal mitochondria and causes cell death. Recent research further shows that Mn stimulates mitochondrial H₂O₂ production over the entire range of concentrations

from those required for normal physiology to concentrations causing cell death [4]. Thus, critical questions exist concerning the mechanisms underlying the Mn-dependent H_2O_2 production, the consequent effects on the redox proteome and the role in mitochondrial signaling and control.

In the simplest case, we can think of Mn as binding to specific macromolecular sites and having specific catalytic, structural or regulatory function. In reality, however, we must view Mn as a divalent cation, competing with other divalent cations for binding and transport. We must question the range of free Mn concentration in different organelles, and the binding of Mn in complexes with different chemical and catalytic activities. We must question the function of Mn in enzymatic as well as non-enzymatic production of oxidants. We must consider the role of Mn and complexes of Mn as an antioxidant, functioning as superoxide dismutase mimetics. These issues are not prioritized because Mn is 139th on an ATSDR list, but rather because every human consumes Mn on a daily basis, and there is little understanding of the contribution of this natural exposure to mitochondrial redox homeostasis, cell survival and cell death signaling.

3. Chemical properties and interactions of Mn with biological molecules

Mn is a trace mineral commonly found in the earth's crust and throughout nature. Mn is often mined in conjunction with other metals such as Fe, and in the periodic chart of elements, Mn is adjacent to Fe in the transition metal series. Mn has numerous applications in commerce, both in metallurgy, such as in the manufacture of steel, and as a powerful oxidizing reagent. Like Fe, Mn exists in multiple oxidation states, and this property is important in its commercial applications as well as biological functions. For simplicity, we use the element symbol without charge state to refer collectively to all forms and symbols with superscripts to refer to specific ion forms. Mn has eleven possible oxidation states that range from Mn^{-3} to Mn^{+7} , with Mn^{+7} having the strongest oxidation state of the group [5]. The predominant oxidation states in biological systems are Mn^{+2} and Mn^{+3} , with the latter having a more positive potential [6, 7].

Because Mn is redox active, present in the environment and transferable to humans, Mn is an important component within the redox interface of an organism and its environment [8]. The concept of a redox interface between the genome and exposome [8] has been elaborated in a set of principles governing the redox organization of aerobic organisms, the Redox Code [9], and extended to address redox changes with aging [10] and the organizational structure of the cysteine proteome [11]. These papers provide background to the consideration of Mn as a key element of this interface and underscore the critical and complex nature of Mn in biological function and failure of redox systems in disease.

Mn has many important roles in biology as a required cofactor and activator of enzymes, especially those responsible for proper bone development, cell structure, metabolism, mitochondrial antioxidant systems, and cell death [12–15]. Examples of these enzymes include galactosyltransferase [16], agmatinase [17], arginase [18], glutamine synthetase [19], pyruvate carboxylase [20, 21] and SOD2 [22, 23], as well as several kinases, hydrolases, transferases, and decarboxylases [24]. Some of these enzymes, such as

manganese-containing SOD2, require a specific oxidation state in the binding pocket to perform its enzymatic reaction. Mn also participates in Lewis acid-base reactions when incorporated in enzymes such as arginase or plays just a structural role to maintain a specific conformation as seen in pyruvate carboxylase [25, 26].

Mn interacts with several other biologically relevant metals such as Fe, Cd, Mg or Ca, by competing with or substituting for these metals, and thereby altering biological functions of these metals [27–31]. Similarly to Mn, these metals also exist in both an elemental and ion forms with multiple oxidation states. For example, Fe exists in biologically relevant ion forms with different oxidation status, Fe^{+2} and Fe^{+3} . In addition, Mn in combination with other metals either synergistically or antagonistically has been demonstrated to induce oxidative stress, cellular damage and apoptosis [32–39]. Mn is structurally and chemically similar to Fe, as seen in Fig. 1, allowing for similar reactions by substitution of the Fe for Mn, although the activity for the substituted enzyme can be highly variable. Other metals of interest, such as Ca^{+2} and Mg^{+2} , have also been found to compete with Mn^{+2} , and it has been observed that as Mn^{+2} concentrations increase, Mg absorption slightly decreases, indicating an inverse relationship [40]. Ca levels were increased as Mn concentrations increased, implying a positive correlation. Other heavy metals, such as Cd, have toxicities decreased upon co-administration with Mn, perhaps due to effects on Cd absorption [41, 42].

The interaction between Mn with these other metal ions is supported by previous findings that Mn shares transport and trafficking mechanisms of other metals. Mn is transported into the cells through the divalent metal transporters DMT1, ZIP8, voltage-gated and store-type calcium channels, and through transferrin receptor-mediated endocytosis (Fig. 2) [43–50]. Transferrin, a well-characterized trafficking protein for Fe, binds and transports Mn^{+3} across the membrane by the same mechanism [51, 52]. For each of these systems, specificity exists for the transport of Mn, depending on the oxidation state, e.g., DMT1 transports Mn^{+2} and transferrin transports Mn^{+3} [45, 47, 51–54].

The multiple transport systems for Mn, as well as competition of Mn for binding to macromolecular structures and potential for electron transfer reactions, introduce concepts that are frequently ignored in discussions of biochemical pathways. To address such complexity, network models are required [55]. These differ from linear pathways in that variable numbers of connections, termed “edges”, can connect the redox active elements, which are termed “hubs”. Maps connecting Mn with functional pathways, redox proteomic networks, transcriptome networks and metabolic networks, are not yet available. These will be important to provide insight into Mn as a component of the redox interface and properly locate Mn within the redox organizational structure of aerobic organisms.

4. Optimal Mn intake range is relatively narrow

Mn is an essential nutrient for mammals. For humans without occupational exposures, the primary source of Mn is through the diet; Mn-rich foods include vegetables, nuts and seafood [56]. Once ingested, less than 5% of Mn is absorbed into the body from the gut [57, 58]. The lower limit for Mn intake has been estimated to be 0.74 mg/d; however, when adjusted for lowest individual percentage retention, the lower limit increases to 2.1 mg/d

[59] (Fig. 3). The recommended dietary allowance (RDA) for Mn is 2.3–5 mg/d [60, 61]; in developing Dietary Recommended Intakes (DRI) in 2001, the Food and Nutrition Board concluded that there was insufficient data to set an Estimated Average Requirement (EAR). They set an Adequate Intake (AI) at 1.8–2.3 mg/d for adult women and men, respectively [62]. A Tolerable Upper Intake Level (UL) of 11 mg/day was set for adults based on a no-observed-adverse-effect level for Western diets and extrapolated data for humans from studies in rats; they calculated the lowest observable adverse effect level (LOAEL) in humans to be 15 mg/d [63, 64].

Other modes of exposure to Mn also occur. Among these, absorption through the skin does not appear to be of great importance, but inhalation occurs in occupations such as welding, steel work, and mining [65]. Such occupational exposures can lead to Mn toxicity, exhibited as a Parkinson-like disease termed “manganism” [66, 67]. In occupational exposure, there is a possibility that Mn is transported to the brain directly from nasal passages in association with the olfactory nerve tracts. While clearly of great importance as an occupational disease, occupational hygiene can largely prevent this exposure. Of potentially greater concern, there is little understanding of the variability of Mn intake in the general population at the usual intake levels. With such a narrow dose range between inadequate and excess intake (Fig. 3) and only 5% oral absorption, small variation in absorption (to 2.5% or 10%) could substantially change the body burden.

A defined LOAEL has been difficult to ascertain due to the limited studies on oral administration of Mn toxicity. Mn accumulates predominantly in the bone; however, other organs such as the liver, kidneys, and the brain also store Mn [68–71]. Chronic exposure to Mn eventually leads to neurotoxicity and Parkinson disease-like symptoms [29, 63, 72–76]. Mn has a half-life of 13–74 d in humans [50, 77]. Mn loss from the body occurs principally via transport into bile and fecal elimination [64, 78]. A relatively recent change in health practices has occurred through inclusion of Mn in over-the counter nutritional supplements. To date, however, few controlled studies of effects of these supplements are available; additionally, a single case of associated manganese overdose has been reported [79]. This can be interpreted to mean that homeostatic measures are sufficient to control Mn absorption to prevent adverse effects or that such effects have not been observed because appropriate end points have not been studied in detail. Of considerable importance, a focus on neurotoxicity of Mn, which is easily justifiable, has led to a relative deficiency in knowledge of Mn effects in other organ systems. Thus, whether expression or activity differences occur for Mn in different organs or under different developmental or disease states is largely unknown. Such descriptive information is fundamentally important to understand the potential roles of Mn in non-neuronal toxicities.

5. Excess Mn causes apoptotic cell death

As summarized above, Mn is essential as a cofactor for multiple enzymes and has the ability to displace other metals in crucial enzymes, alter metal transport and induce oxidative stress, thereby disrupting a number of processes. While the exact mechanism of action for Mn dependent cellular death remains uncertain, multiple apoptosis-related genes, proteins and processes are induced in response to Mn treatment (Fig. 4). As indicated above, exposure to

high levels of Mn causes manganism, with neurological symptoms similar to Parkinson's disease (PD) [80–82]. Apoptosis plays an important role in a progressive loss of dopaminergic neurons in PD [83–86]. Several groups have demonstrated in model systems that exposure to Mn results in cellular cytotoxic events and apoptotic signaling. Briefly, SH-SY5Y human neuroblastoma cell line exposed to 0.5–1.0 mM MnCl₂ for 24 h, caused apoptotic cell death rate to increase to 11% and 16% respectively, compared to control cells at 4% [87]. PC12 cells exposed to 0.1 mM MnCl₂ for 24 h, demonstrated induced c-Jun N-terminal kinase signaling and hallmarks of apoptosis characterized by inter-nucleosomal DNA fragmentation [88].

Mn induces caspase-dependent apoptosis (Fig. 4) [15, 89–94]. Caspases are endoproteases that initiate (8 and 9) and execute (3, 6 and 7) apoptotic events [95]. Cell death pathways through caspase-3 cleavage seem to be the predominant mode of Mn-dependent caspase-induced apoptosis in neuronal cells [90, 92, 94, 96, 97]. In addition to caspase-3 cleavage, Mn also increases caspase 3-promoter activity through the Sp1 binding regions in PC-12 cells treated with 0.25 mM–1mM MnCl₂ for 18 h, thereby increasing caspase-3 mRNA and proteins (Fig. 4) [90].

Induction of Mn-induced cell death pathways have also been linked to oxidative stress and oxidant species generated by Mn treatment [98–102]. PC12 cells treated with 2 mM MnCl₂ for 36 h demonstrated increased rate of apoptosis related to increased oxidant production and mitochondrial dysfunction [99]. Treatments with antioxidants prevent Mn-induced apoptotic events and thereby indicate the role of oxidants in Mn cytotoxicity [103, 104]. Our recent study [4] shows that mitochondria in SH-SY5Y human neuroblastoma cells treated with Mn (0–100 μM, 5 h) increased mitochondrial H₂O₂ production in a dose dependent manner; superoxide anion radical may also be increased, however a concomitant increase in SOD2 activity also occurred so that no apparent increase in superoxide was detected [4].

The exact origin of Mn-induced cell death signal still remains obscure and may be dependent on multiple factors such as the cell type, extent of exposure or susceptibility of the organ. Organelles involved in Mn-induced apoptosis include mitochondria [91, 105–109], endoplasmic reticulum [93, 110, 111] or both [112, 113]. As indicated below, molecular tools to monitor free and/or bound redox-active Mn pools are limited. Thus, the relative concentrations, associated transport and protein binding, in different organelles, is poorly understood.

While most of the research studies have focused on Mn-dependent neuronal dysfunction, induction of apoptosis by Mn appears to be a global property. The organ priority of Mn toxicity and impact vary, but evidence shows that Mn exhibits apoptotic properties in several cell types [15, 89–94, 114, 115]. For instance, human bronchial epithelial (16HBE) cells exposed to MnCl₂ for 12 or 24 h exhibited caspase-9 dependent cell death along with caspase-3 cleavage, the down regulation of c-Myc and the up regulation of p53 and phosphorylated p53 [89]. Treatment of human B cells with 0.4 mM MnCl₂ for 24 h induced caspase-8-dependent apoptosis, probably through the sequential activation of p38 MAPK, MSK1, caspase-8 and the mitochondria [91]. Thus, more specific comparative studies will be needed to evaluate contributions of Mn to cellular homeostasis in different organ systems.

This will be especially important in the context of health and disease, but the results showing increased H_2O_2 production as a function of Mn across the entire physiological to toxicological range [4] suggests that Mn concentration could impact other toxicities involving mitochondrial oxidative mechanisms.

6. Antioxidant as well as prooxidant roles of Mn in redox biology

Mn-related cellular toxicity is associated with increased production of oxidants and mitochondrial dysfunction ([94, 104, 116–122]. Mn also plays a very important role, however, in the mitochondrial antioxidant defense. Mn-dependent superoxide dismutase (SOD2) was found in the matrix space of mitochondria [123, 124] and utilizes Mn to dismutate superoxide ($\text{O}_2^{\cdot -}$) to H_2O_2 and O_2 (Fig. 5). SOD2 is a key enzyme in cellular defense against oxidative stress. Mitochondrial SOD2 is translated on cytoplasmic ribosomes and imported into the mitochondria with a mitochondrial targeting sequence of 26 amino acids. This targeting sequence is cleaved, leaving a 22 kD monomer, which incorporates Mn^{+3} and assembles into an 88 kD fully functional homotetramer [125–128]. The balance of Mn^{+2} and Mn^{+3} concentrations in mitochondria is not well understood [129] and similarly, it is unclear whether specific chaperones function in this process.

In the catalytic turnover, Mn in SOD2 alternates between Mn^{+2} and Mn^{+3} states. Some forms of bacterial Mn-dependent SOD incorporate Fe instead of Mn, dependent on metal availability [127]. In yeast, Fe binds to SOD2 when levels of mitochondrial Mn or Fe are disrupted, and results in an inactive enzyme [130, 131]. Mammalian SOD2 activity is highly specific for Mn and inactivated by binding with Fe [132, 133]. The redox potential of the SOD2 metal site (+200 to +400 mV) lies between that of oxygen/superoxide (– 160 mV) and superoxide/hydrogen peroxide (+890 mV), making SOD2 highly effective in eliminating superoxide. Fe binding in place of Mn shifted the redox potential to more negative value (– 200 mV), thereby making it less efficient to complete superoxide dismutation; this effect may account for the evolutionary selection of Mn over Fe incorporation in SOD2 in mammals [134]. Regardless, the key point is that mitochondria require Mn for this important function, interconnecting mitochondrial superoxide with H_2O_2 and O_2 . In this regard, Mn not only functions to maintain superoxide levels but also contributes to H_2O_2 homeostasis and redox control [135].

Together with the knowledge that excess Mn causes oxidative stress, this role in maintenance of the superoxide concentration places Mn as a critical component of the redox interface between an organism and its environment. The redox activities of Mn support beneficial effects in redox systems control and harmful effects in disruption of redox systems when present in excess. Variation in Mn concentrations within cells could therefore globally impact the redox environment of the cell. In the context of the Redox Code [9], Mn may be an unrecognized hub within the global redox network structure. Hence, systematic variation in Mn exposure with detailed examination of redox proteomics and related transcriptional and metabolic effects will be needed to more fully understand Mn function within redox network structures.

7. Mn in subcellular compartments

Understanding the distribution of Mn among subcellular compartments in different cell types represents a challenging research problem. Insufficient numbers and representative cell types have been studied to arrive at generalizations concerning involvement of multiple transporters, binding proteins and competition with other metals. Despite this, quality studies are available to show that Mn has important roles in mitochondria, nuclei, golgi and endoplasmic reticulum. Even though additional research is needed, useful information is available to help direct future studies.

Mitochondrial localization of SOD2 highlights mitochondria as a target for Mn uptake, and toxicologic studies on the subcellular distribution of Mn demonstrate that treatment with Mn leads to mitochondrial Mn accumulation [136–138]. In the mitochondria, Mn-induced toxicity is characterized by the inactivation of multiple mitochondrial enzymes (especially the ones that participate in oxidative phosphorylation and Krebs cycle), oxidant generation and disruption of mitochondrial membrane potential (Fig. 4) [122, 139–141]. In the microglia, Mn-induced H₂O₂ production appears to be mediated through mitochondrial complex II [142, 143]. In the liver and heart mitochondria, Mn was found to inhibit F₁F₀ ATP synthase, which is also a Ca⁺² activated protein [139]. Occupancy of Mn in Ca⁺² binding sites could further exacerbate oxidant production through disrupted Ca⁺² homeostasis. *In vitro* and *in vivo* studies show chronic Mn exposure significantly inhibits mitochondrial aconitase, an enzyme whose activity is susceptible to oxidative stress owing to its iron-sulfur center. Loss of aconitase activity disrupts energy production and also iron homeostasis [144, 145].

Radiotracer studies also show that Mn accumulates in the nuclei of cultured brain cells, including Z310, RBE4, N27 and PC12 cells [146], and also in rat liver cell nuclei [147, 148]. Multiple *in vitro* studies have demonstrated Mn binding to DNA, RNA and ribosomes [149, 150] resulting in transcriptional and translational dysregulation (Fig. 4). Furthermore, Mn at 2 and 62 µM for 24 h induced the formation and accumulation of DNA single strand breaks and oxidatively modified thymine bases in SHSY5Y cells. These effects were attenuated by treatment with the thiol antioxidants N-ace-tylcysteine or glutathione [151]. However, whether Mn was directly associated with the lesions or indirectly through oxidant species generated in other organelles is unknown because the nuclear Mn and nuclear oxidant production were not measured.

Golgi helps in Mn detoxification in yeast and plants [152, 153], and Synchrotron X-ray fluorescence nanoimaging of PC12 dopaminergic cells revealed that manganese is located within the Golgi apparatus at physiological concentration and increased with increasing concentrations [154]. The increase in Mn concentrations further results in higher accumulation of Mn in the cytoplasm, suggesting that under physiological concentrations, Golgi may help in Mn detoxification. Alternatively, Mn may have a function in the Golgi that is yet to be identified.

Mn-induced endoplasmic reticulum (ER) stress response has been observed with alterations in ER stress gene and protein levels (Fig. 4) [38, 155, 156]. *In vitro* inhibition of ER stress

by the chemical chaperone 4-phenylbutyrate fully reversed Mn-induced cytotoxicity, indicating a role of ER in Mn toxicity [38]. Depletion of Fe increased cellular uptake of Mn and induced ER stress, thus highlighting the role of metal dynamics in cellular toxicity [38]. Interestingly, the ER unfolded protein response (UPR) leads to apoptosis through mitochondria-linked and mitochondria-independent mechanisms [157, 158]. Further studies are needed to determine whether Mn activates the UPR to trigger apoptosis directly or through other Mn-dependent activities [159].

8. Methods for quantitative analysis of Mn

Because of the importance of manganese in cellular function, it is important to know the levels of manganese in biological matrices, such as blood, or tissue. Two of the most well characterized methods to detect the levels of intracellular Mn are Zeeman graphite furnace atomic absorption spectroscopy (GFAAS) and inductively coupled plasma mass spectrometry (ICP-MS) [160–162]. Both of these methods utilize high temperature to generate requisite ionic species for detection and quantification. Also, these methods are destructive, meaning that they cannot be used on living cells or organisms.

Mn-Enhanced Magnetic Resonance Imaging (MEMRI) is a technique, which allows the mapping and quantification of Mn^{+2} ions in the brain [163, 164]. It is applicable to *in vivo* studies in experimental animals and is being developed for human applications. Additional methods to measure Mn in live cells and subcellular compartments are needed. Indirect measure of Mn^{+2} concentrations have been obtained with the Cellular Fura-2 Manganese Extraction Assay (CFMEA), which utilizes the fluorescent probe Fura-2 with excitation and emission at 360 nm, and 535 nm, respectively [165]. Methods utilizing X-ray fluorescence microscopy have also been developed to determine and quantify the oxidation state of Mn within tissues [166]. These methods in combination enable quantification of the total amount of Mn, its localization, and its oxidation state(s) [167]. Additional application of these methods is expected to support progress in understanding molecular details of the redox biology of Mn, but other tools, such as ratiometric fluorescent probes specific for Mn, are needed to target measurements in subcellular organelles and allow imaging of Mn concentration in living cells.

9. Manganese-based compounds for industrial and therapeutic uses

As indicated above, Mn has wide use in industry as a useful catalyst, pesticide, and automotive fuel enhancer. For example, Mn dioxide (MnO_2) oxidizes numerous compounds such as aromatics and alcohols [168, 169]. Compounds like maneb or mancozeb, both Mn containing fungicides, have been widely studied in toxicities and associated mechanisms [170, 171]. Lastly, methyl-cyclopentadienyl manganese tricarbonyl (MMT) is an octane enhancer added to gasoline in many countries. Toxicity concerns have been expressed because MMT has an LD_{50} of 50 mg/kg in rats [172] and emitted Mn is potentially neurotoxic. Available evidence is equivocal, however, that Mn is toxic at exposures levels occurring with gasoline combustion. This remaining controversy emphasizes the critical nature of understanding potential adverse effects of Mn at low levels to which humans are exposed.

While Mn-induced cell death may contribute to disease, Mn-induced cell death has also been explored as a potential cancer therapeutic strategy. Mn complexes such as Mn porphyrin, [MnTE-2-PyP⁵⁺] increase glucocorticoid-induced apoptosis in WEHI7.2 murine thymic lymphoma cells [173]. Another recently synthesized Mn complex, AdpaMn [(Adpa)Mn(Cl)(H₂O)] (Adpa = bis(2-pyridylmethyl)amino-2-propionic acid) increased apoptosis of malignant glioma cells, U251 and C6 cells. In these cancer cells, the Mn complexes targeted mitochondria and increased oxidants; importantly, the complexes did not affect normal astrocytes [105, 174]. Cefepime complexed with Mn was found to be most potent among other metal complexes in inhibiting proteosomal activity and inducing apoptosis in MDA-MB231 human breast cancer cells. Normal breast epithelial cells MCF-10A were much less sensitive to this complex [175]. MnNG (Mn-(II) N-(2-hydroxy acetophenone) glycinate) induced apoptosis in doxorubicin resistant human T lymphoblastic leukemia cells (CEM/ADR 5000).

The use of Mn porphyrins in cancer therapeutics involves combination with ascorbate to create a powerful redox system that preferentially kills cancer cells. Such combination shows efficacy in inducing apoptosis in cancer cells [176]. *In vivo* studies with intraperitoneal application of non-toxic dosage of MnNG significantly increased the life-span of Swiss albino mice bearing sensitive and doxorubicin resistant subline of Ehrlich ascites carcinoma cells [177]. Such combination of essential antioxidant vitamin with an Mn complex to cause apoptosis further illustrates the characteristic of Mn in the redox interface of an organism with its environment. Two otherwise essential nutrients, Mn and ascorbate, selectively kill cancer cells when placed in this therapeutic context. Whether analogous combinations occur naturally and contribute to abnormal cell death and disease remains unknown.

10. Future research for Mn associated with cell death and toxicity

As emphasized throughout this review, Mn plays an important role at the redox interface between an organism and its environment. The chemistry of Mn supports a critical role as a redox regulator through its activity of SOD2. Through excessive accumulation or disruption of Mn homeostasis, Mn initiates apoptotic signaling and directly functions in neurodegenerative disease. As we look to the future for Mn research, there are a number of important needs.

Most critically, there is a need to understand the redox network responses to varied Mn exposure. Mn causes increased mitochondrial H₂O₂ production over the entire physiological to pathological range [4]. How this increased H₂O₂ production impacts the redox proteome is currently unknown. Because cysteine residues within proteins undergo continuous oxidation and reduction, Mn-dependent production of H₂O₂ is expected to impact redox-sensitive protein systems. Oxidation of the mitochondrial proteome alters carnitine-acyl transferase activity and disrupts fatty acid β -oxidation [178]. Such changes in metabolome are also accompanied by changes in the transcriptome [179]. Thus, combined studies of the redox proteome, metabolome and transcriptome as a function of Mn concentration provides a way to build upon the finding that mitochondrial H₂O₂ production depends directly upon Mn exposure.

The multiple types of interactions that Mn has with other metals and metal-dependent systems associated with cell death signaling also warrants further study. The modes of action depend upon exposure dosage; because other metals are present and can impact transport and protein binding, the sites and mechanisms of Mn toxicity cannot be fully understood without assessment of other metals. Accordingly, more detailed metallomics approaches involving measurements of many metals will aid in understanding Mn toxicity. ICP-MS facilitates measurement of multiple metals in biological samples and enables more powerful, in-depth studies. Coupled with this, however, is a need to incorporate systems biology approaches to address the complexities of Mn signaling, especially to understand the impacts of Mn on apoptosis within the metal mixtures in diverse tissues.

Human exposure to Mn is mostly derived from the diet. Surprisingly, less than 5% of dietary Mn is absorbed. Because the biological half-life is weeks to months, changes in efficiency of absorption or elimination could have substantial effects on the body burden. Complexes of Mn in the diet are not well characterized, and studies to examine the interactions of Mn with other metals and the intestinal microbiome are needed. Because the primary mechanism for Mn elimination involves hepatic secretion into bile, there is also a need to understand factors impacting Mn secretion and subsequent loss.

As new methods and technologies become available, the sensitivity for Mn detection and quantification is expected to improve. Improved detection of Mn complexes will help address the possible roles of Mn-complexes in disease. In addition, the ability to quantify in different subcellular compartments, such as the levels of Mn in the nucleus, a previously unexplored compartment, will also become possible. Such capabilities for subcellular quantification of Mn will facilitate important studies of toxicological mechanism. With improved detection methods, the ability to track the movement of Mn among subcellular compartments will become possible. As described above, the mechanisms of Mn in apoptotic signaling remain unclear. For instance; Mn could activate nuclear apoptotic signaling that is distinct from mitochondrial or ER systems. Additional research is needed to more fully understand Mn-dependent downstream signaling cascades.

Recent development of Mn-enhanced MRI in human brain provides new opportunity to test for manganese due to work exposure by providing the ability to detect Mn *in vivo* [180]. This approach quantifies Mn⁺²; therefore, detection of *in vivo* levels of Mn⁺³ or other oxidation states remains under development. An additional need is capability to distinguish total, free and bound forms. Additionally, different oxidation states remain undetermined; if Mn is transported and utilized like Fe, these forms could be very important. As these studies further develop sophisticated methods to detect and quantify the different forms of Mn *in vivo*, evaluation of relatively weakly bound Mn to the surface of proteins may become possible. Whether such binding of redox-active metals, such as Mn, to surface thiols functions in cellular redox control remains an important area of exploration [181, 182].

Much of the Mn literature to date is on the role of Mn in central nervous system or neuronal cell lines. However the lungs are a primary site of Mn exposure, and lungs and other organs are susceptible to Mn effects. At least some of the central pathways in neurons are shared

among other tissues, and studies are needed to evaluate how Mn alters the metabolic landscape after chronic administration.

Thus, we can visualize questions, which we believe Sten Orrenius would find interesting. What are the common mechanisms by which Mn impacts health and disease? What are the molecular targets, and is there a hierarchy among the targets? What are the most powerful methods to address the complex interactions of Mn with the molecular machinery of life while also accounting for metal-metal interactions and Mn binding to small molecules? Can we use the latest integrative approaches, such as metabolomics, proteomics, and transcriptomics, to understand the role of Mn on the fate of the cell. As we look towards the future, we must never forget the fundamental principles that Sten Orrenius gave us: engage our scientific colleagues, think simply and clearly about the most fundamental questions, enjoy the process of discovery and communication of findings, and especially, be satisfied with the rewarding life as a scientist.

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Fig. 1.

Manganese is similar to iron due to comparable electron shells and atomic radii. Elemental manganese has 13 electrons in the 3rd orbital (A, orange) in comparison to Fe which has 14 (B, dark orange). Both Mn and Fe share 2 electrons (e^-) in the 4s orbital (black).

Comparing the different oxidation state of electron configurations between Fe and Mn, in Fe²⁺ both electrons in the 4s orbital are lost, however there remains a paired set of electrons in the 3d orbital. Pairing of other 3d electrons can occur in the presence of strong field ligands (B, Fe²⁺). Fe³⁺ ions (B, Fe³⁺) share the same number of electrons as Mn²⁺ (A, Mn²⁺) with 5 unpaired electrons in the 3d orbital compared to Mn³⁺ which has 4 unpaired electrons in the 3d orbital (A, Mn³⁺). In addition, comparing the atomic and ionic radii of Mn (A, bottom) and Fe (B, bottom) also shows similarity in the size of the orbitals using 6-coordinate, low-spin, octahedral complexes.

Fig. 2.

Common Mn transporters in the cell. A, Transferrin Receptors (TfR) bind to transferrin which has been complexed to Mn^{+3} , Fe^{+2} , or Cu^{+2} . The membrane undergoes endocytosis releasing Mn^{+3} from transferrin where Mn^{+3} is reduced to Mn^{+2} and pumped out of the vesicle by the divalent metal transporter 1 (DMT1). B, DMT1 transporters pump many divalent metals such as Cd^{+2} , Co^{+2} , Fe^{+2} , Mn^{+2} , Ni^{+2} , Pb^{+2} , and Zn^{+2} across the membrane. C, The voltage gated ZIP8 channels also pump Cd^{+2} , Co^{+2} , Fe^{+2} , Mn^{+2} , and Zn^{+2} across the membrane. D, Na^{+} and Mn^{+2} competitively bind to the choline transporter across the membrane. E, The metal-citrate transporter functions by citrate binding to metals such as Ca^{+2} , Co^{+2} , Fe^{+3} , Mg^{+2} , Mn^{+2} , Na^{+} , Ni^{+2} , and Zn^{+2} allowing transport across the membrane. Lastly the Ca^{+2} transporter is a divalent metal transporter (F) which can also transport Ca^{+2} , Cd^{+2} , Mn^{+2} , and Zn^{+2} across the membrane. These transporters are found in multiple organellar membranes including plasma membrane (PM), nuclear membrane (NM), mitochondrial membrane (MM) and golgi membrane (GM). Note; the representative metals but not all are indicated for transporters.

Fig. 3.

Mn intake and adequate dose range for individuals. Mn absorption is critical to maintain healthy function in all individuals. In Mn-deficient individuals, problems such as impaired growth, skeletal and bone defects, abnormal glucose tolerance, and increased oxidative stress and mitochondrial dysfunction have been observed [59, 183, 184]. The daily recommended intake (DRI) has been found to be 1.8 mg/d for females and 2.3 mg/d for males which is also close to the Low Mn intake level. The upper limit for Mn absorption has been found to be 11 mg/d. Mn toxicity occurs above the upper limit, and toxicity has been calculated to begin at 15 mg/d. Other consequences of excess Mn absorption in addition to apoptosis include manganism, Mn-induced PD, other neurological disorders, increased oxidative stress and mitochondrial dysfunction [80–86].

Fig. 4.

Proposed diagram of Mn-induced apoptosis. As Mn accumulates within different organelles in the cell, differing responses can initiate differing caspases in order to activate apoptosis. In the mitochondria, excess Mn causes increased oxidants which leads to cytochrome c (Cyt-c) release and increased transcriptional disruption. This Cyt-c release leads to caspase 8 and 9 activation which activates caspase 3 initiating apoptosis. Mn accumulation decreases membrane potential ($\Delta\Psi_m$) and ATP while increasing mitochondrial fission and decreases mitochondrial fusion regulated by Drp-1 and Opa-1, respectively. Mn accumulation in the cytosol can lead to PKC δ activation which also activates caspase 8 followed by activation of caspase 3 to initiate apoptosis. Mn accumulation in the nucleus can lead to chromatin condensation, DNA fragmentation, and increased mRNA levels of caspase 3. Lastly, Mn accumulation in ER leads to increased ER stress, and increases unfolded protein response proteins such as Bip, PERK, Bim and Bax among others. These proteins can initiate caspase 12 activation which also leads to apoptosis through caspase 3. Abbreviations; Drp-1, Dynamin related protein; Opa-1, dynamin like 120 kDa protein, PKC δ , protein kinase c delta; Bip, binding immunoglobulin protein; p-IRE-1, phospho-inositol requiring enzyme 1; PERK, protein kinase RNA-like endoplasmic reticulum kinase; Σ 1R, sigma-1R receptor; CHOP, C/EBP homologous protein; Bim, Bcl-2-like protein 11; Bax, Bcl2-associated X protein.

Fig. 5.

Role of Mn in enzymatic activity. A, SOD2 is found within the mitochondria and catalyzes the dismutation of superoxide into molecular oxygen and Mn^{+2} . Mn^{+2} (gray) can then be oxidized into Mn^{+3} (orange) through the catalysis of another molecule of superoxide into hydrogen peroxide. B, In pyruvate carboxylase, Mn^{+2} interacts with water to stabilize oxaloacetate (OAA) and oxygen from GTP to allow the conversion of OAA to pyruvate (PYR), leading to the conversion of PYR to phosphoenolpyruvate (PEP) [185]. C, Arginase utilizes Mn^{+2} as a Lewis acid as opposed to its redox properties to accept electrons from water allowing the formation of a hydroxide molecule.