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Decreased expression of inflammation-related genes following inhalation exposure to manganese

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Abstract

Excessive exposure to manganese (Mn) by inhalation can induce psychosis and Parkinsonism. The clinical manifestations of Mn neurotoxicity have been related to numerous physiological and cellular processes, most notably dopamine depletion. However, few studies have explored the molecular events that are triggered in response to exposure to Mn by inhalation. In this current study, the transcriptional patterns of genes related to oxidative stress or inflammation were examined in the brain rats of exposed to inhaled Mn during either gestation or early adulthood. The expression of genes encoding for proteins critical to an inflammatory response and/or possessing pro-oxidant properties, including TGF β and nNOS, were slightly depressed by prenatal exposure, whereas inhalation exposure to Mn during adulthood markedly down-regulated their transcription. However, when exposures to manganese occurred during gestation, the extent of altered gene expression induced by subsequent exposure to Mn in adulthood was reduced. This suggests that prior exposure to Mn may have attenuated the effects of inhalation exposure to Mn in adulthood, in which the expression of inflammation-related genes were suppressed.

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Keywords: Manganese; Developmental neurotoxicology; Inhalation exposure; Gene expression; Inflammation; Nitric oxide synthetase; Heat shock proteins

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1. Introduction

Mn toxicity in humans primarily occurs as a consequence of chronic inhalation of high concentrations of airborne Mn-containing particles. Symptoms of irreversible neurological dysfunction similar to Parkinson's disease have been observed in miners, ferroalloy and battery manufacture workers, and automotive repair workers exposed to airborne particles containing Mn (Takeda, 2003; Wennberg et al., 1991). Studies of more recent outbreaks of toxicity arising from occupational exposures to Mn have measured concentrations in the range of 5 mg Mn/m³ with particle sizes of less than 5 μ m. However, clinical indications of Mn toxicity from chronic exposure to concentrations as low as 1 mg Mn/m³ have been reported in

surveys of workers exposed to Mn (Iregren, 1990; Roels et al., 1987; Wennberg et al., 1991).

In addition to occupational settings, environmental levels of airborne Mn have also been proposed to be a health risk to the general population. In urban areas, industrial point sources and other anthropogenic sources, such as fossil fuel combustion, incineration of wastes, and road dust can contribute significantly to ambient levels of Mn, which range from 65 to 166 ng/m³ on average. However, near source-dominated regions, the air concentration can reach concentrations of 8000 ng/m³ (Stokes et al., 1988). Geographic analyses of those living in southwestern regions of Quebec revealed a positive correlation between higher levels of airborne Mn and increased levels of Mn in the blood (Baldwin et al., 1999). The mean total-particulate airborne Mn concentration for the regions was calculated to be 0.022 μ g/m³, roughly half that of the EPA reference concentration (IRIS, 1993).

Although outbreaks of manganism may occur from the oral intake of high Mn levels, the inhalation route is a far more effective pathway of entry for Mn and accounts for the more severe cases of neurotoxicity (Normandin et al., 2002). This

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higher efficiency arises from the greater capacity of the lungs to absorb and retain Mn for transfer to the circulation (Andersen et al., 1999), and the direct uptake of Mn salts from the nasal passages to the brain (Tjalve et al., 1996). The significance of the inhalation route of exposure in Mn-induced neurotoxicity has been long established by experimental evidence (van Bogaert and Dallemagne, 1946).

Despite this, decades of research have used oral or intraperitoneal administration to relate numerous physiological and cellular processes to Mn neurotoxicity. There are very few reports of the effects of Mn inhalation on gene expression in the brain (Dobson et al., 2003; Erikson et al., 2005). In the current study, the transcriptional patterns of stress genes considered critical to an inflammatory response were compared in the brains of rats exposed to Mn during either gestation or early adulthood by inhalation. In addition, the effect of an initial gestational exposure to Mn, followed by a subsequent exposure in early adulthood, was studied.

2. Experimental procedures

2.1. Animal husbandry

A total of 12 timed-pregnant female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA), and maintained in the vivarium at UC Irvine, Air Pollution Health Effects Laboratory. Females among the progeny were selected after weaning at 22 days post-delivery and randomly divided into four groups with six animals in each group, and housed in stainless steel cages with hardwood-chips bedding. Animals were housed in an AAALAC-accredited vivarium, in which room conditions of a 12-h light/dark cycle, a stable ambient temperature of 22 °C, and a humidity level of 70% were maintained. Lab chow consisting of 10–15 ppm Mn (diet AIN-93G, Dyets Inc., Bethlehem, PA) and distilled water containing less than 1 ppb Mn were provided ad libitum. Body weight, food, and water consumption were measured weekly. All experiments involving animals were undertaken following the approval of and in accordance with the guidelines of the Institutional Animal Care and Use Committee.

2.2. Aerosol generation

Submicron particle-sized aerosol was generated from distilled water or a 1% MnSO₄ solution (Sigma Aldrich, St. Louis, MO) using a Collison nebulizer, which was equipped with an impactor to remove large vapor droplets from the aerosol. The aerosol was diluted with dry, HEPA-filtered purified air and deionized with an ⁸⁵Kr particle discharger prior to being introduced into the 72-port nose-only exposure manifold (In-Tox, Albuquerque, NM). Aerosol flow into the manifold was maintained under slight positive pressure to minimize possible contamination of the exposure atmosphere with ambient air. The exposure system manifold was located and exposures conducted in a sealed chamber under constant temperature and humidity control.

2.3. Aerosol characterization

Samples were collected from the breathing zone of the animals to document the exposure particle size and Mn concentration, and to monitor the distribution of test compounds throughout the manifold. The range of aerosol concentration was 2.22 ± 0.14 mg MnSO₄/m³, which is equivalent to a concentration of 0.71 mg Mn/m³. Particle size distribution was assessed with a cascade impactor, which was fitted with multiple mylar stages and orifices (Model 298, Anderson Samplers Inc., Atlanta, G). The mass median aerodynamic diameter was 0.55 μm ($\sigma_g = 1.5$) as determined by gravimetric sampling.

2.4. Inhalation exposure to manganese

Rats were exposed to either aerosolized distilled water or manganese sulfate (MnSO₄), which was delivered using a nose-only inhalation chamber (Kleinman et al., 1999) at two different time points: the 2nd week after formation of the sperm plug (starting at gestation day 9) or the 6th week after birth (starting at postnatal 37). The air was delivered to an annular chamber with baffles to ensure uniform distribution of particles to each nose-only port. Rats were randomized into different ports at each exposure to mitigate exposure biases.

Animals of the control group were administered HEPA-filtered purified air at both time points. Animals of the first experimental group (designated prenatal exposure) were exposed to Mn during gestation, and then administered purified air at 6 weeks after birth. The dams of this group were administered Mn by inhalation for 2-h periods on 2 consecutive days at the 2nd week of pregnancy. Animals of the second experimental group (designated adulthood exposure) were exposed to purified air during the same gestation time point, and then administered Mn at 6 weeks after birth for daily 2-h periods for 10 consecutive days. Those of the third experimental group (designated prenatal + adulthood exposure) were exposed to 2-h treatment with Mn at both time points, i.e. 2-h exposures on 2 days at the 2nd week of pregnancy and then again over 10 days at 6 weeks age.

Three days after the last exposure date (to either purified air or Mn) in young adulthood (postnatal day 49), all animals were killed by decapitation. Brains were immediately removed, rinsed with ice-cold saline, divided into sagittal halves, frozen with dry ice, and stored at –70 °C. The brains of female animals were subject to RNA isolation and protein extraction, whereas the brains of male counterparts underwent neutron activation analysis. Due to the relatively small number of animals included in the pilot study, all of the available brain tissue that was derived from the female animals was allocated for the study of genetic changes. However, it has been reported that gender does not influence the transport of inhaled manganese to the striatum, the primary target site of manganese neurotoxicity, in rats (Dorman et al., 2004a).

2.5. RNA extraction and protein isolation

Brain tissue homogenates were suspended in a mixture of chloroform, phenol and guanidine thiocyanate. Total RNA was extracted from the reagent, and concentrations were determined by absorption at 260 nm wavelength. Purity of the RNA was monitored by measuring the ratio of absorbance at 260 nm to that at 280 nm. Protein was isolated from the same samples, and quantified by Bicinchoninic acid Assay (BCA, Pierce Technologies, Rockford, IL).

2.6. Northern blot analysis

Aliquots of 10 µg total RNA isolated from rat brain were denatured with formaldehyde and formamide, and electrophoresed on 1.2% agarose gel containing 6% formaldehyde. Each gel was loaded with samples from all of the four exposure groups. Each lane of a gel was loaded with a sample derived from a single animal. Thus, representatives of all groups were run simultaneously on each of the gels, facilitating comparison and reducing variability of experimental parameters. The RNA separated by the gel was transferred onto nylon Zeta-probe blotting membranes (Bio-Rad Laboratories, Hercules, CA). Membrane-bound RNA was then hybridized with the cDNA probe specific to the gene of interest, and random prime-labeled with [³²P] dCTP. Each band represented the hybridization of the labeled cDNA probe to the gene transcript. Standardization of RNA loads was verified by gel ethidium bromide fluorescence intensity, as well as by comparison of the presence of housekeeping gene actin-γ. Membranes were autoradiographed for periods varying from 8 h to 7 days at −70 °C on X-ray film. A densitometer (Eagle Eye image-processor and DNA Scan signal analysis software, Stratagene, San Diego, CA) was used to quantify the signals as area-integrated optical density.

2.7. Neutron activation analysis

Concentrations of elemental manganese in brain tissue were determined by Neutron Activation Analysis (NAA) at the UCI Nuclear Reactor Facility Laboratory. Assays were performed on 3 male rats from each treatment group. Samples were dried and heat-sealed into acid-cleaned, polyethylene vials under clean-room conditions to minimize any surface contamination. Irradiation of all samples occurred at a neutron flux of 3×10^{11} neutrons/cm² s for 10–15 min. A period of 1 h was scheduled after irradiation to ensure that the possible presence of ²⁸Mg was decayed to negligible amounts. Radioactive decay of ⁵⁶Mn was measured using a Gamma-ray Spectrometer (30% HPGe detector, Canberra, IN). Sample-detector distance was selected at 1 or 2 cm, depending on activity level. Standard NIST Bovine Liver (SRM 1577) samples were used to calibrate Mn concentrations in each run. Blank vials were assayed to correct for small amounts of Mn intrinsic to the plastic material of the vials. Peak areas were calculated using the Canberra ASAP software package.

2.8. Statistical analyses

The data obtained were statistically evaluated using analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The accepted level of significance was $p < 0.05$ using two-tailed analysis.

3. Results

The rate of gain in body weight and overall appearance of rats were not distinguishable among the different groups, suggesting that the Mn exposures administered in this study caused no major toxic effects. In the brains of rats exposed to

Table 1
List of genes assayed by Northern blotting, and brief descriptions of their known functions

Gene name	Abbreviation	Description
Amyloid precursor protein	APP	Neurotrophic factor; redox-sensitive
Cyclooxygenase-2	COX-2	Neurotrophic factor; redox-sensitive
Glial fibrillary acidic protein	GFAP	Inflammation-related; neurotrophic factor; redox-sensitive
Glutamate cysteine ligase catalytic subunit	GCL-c	Redox-sensitive
Interleukin-1	IL-1β	Cytokine; redox-sensitive
Interleukin-6	IL-6	Cytokine; redox-sensitive
Neuronal nitric oxide synthetase	nNOS	Neurotrophic factor; redox-sensitive
Retinoic X receptor	RXR-α	Neurotrophic factor; redox-sensitive
Transforming growth factor	TGF β	Cytokine; redox-sensitive
Translocator of outer mitochondrial membrane	TOM40	Redox-sensitive

Items highlighted in grey represent genes, which had changed expression in the rat brain following subchronic exposure to MnSO₄ either during gestation and/or adulthood. Genes not highlighted were expressed at insufficient levels to be statistically compared in controls and in Mn-challenged rats.

MnSO₄ either during gestation or adulthood, a set of 10 neural genes that have been associated with oxidative stress and inflammation, was assessed by Northern blotting (Table 1). Mn-induced modulations in levels of mRNA were detectable in five of the genes. The expression levels of the remaining genes were insufficient to quantify with accuracy and thus effects of Mn could not be determined. Genes clearly responding to Mn exposure were those encoding for amyloid precursor protein (APP), cyclooxygenase-2 (COX-2), neuronal nitric oxide synthase (nNOS), glial fibrillary acidic protein (GFAP), and transformation growth factor beta (TGF- β) (Figs. 1–5). The transcription levels of these genes were all significantly

Fig. 1. Transcript levels of gene APP determined by quantitation of autoradiograms of Northern blots. Values were normalized to actin mRNA. Values represent the mean $\pm$ S.E. of three independent determinations of 3–5 animals from each group. (*) Mn-treated animals of this group differ ($p < 0.05$) from animals of other Mn-treated groups and those of the control group.

Fig. 2. Transcript levels of gene COX-2 determined by quantitation of autoradiograms of Northern blots. Values were normalized to actin mRNA. Values represent the mean $\pm$ S.E. of three independent determinations of 3–5 animals from each group. (*) Mn-treated animals of this group differ ($p < 0.05$) from animals of other Mn-treated groups and those of the control group.

Fig. 3. Transcript levels of gene nNOS determined by quantitation of autoradiograms of Northern blots. Values were normalized to actin mRNA. Values represent the mean $\pm$ S.E. of three independent determinations of 3–5 animals from each group. (*) Mn-treated animals of this group differ ($p < 0.05$) from animals of other Mn-treated groups and those of the control group.

decreased following exposure to Mn only during adulthood. There was a lesser decrease of expression of these genes in animals belonging to either of the two groups exposed to Mn during gestation (prenatal, or prenatal + adulthood exposure). For animals treated prenatally only, this depression was significant for expression of all genes studied except TGF- β . While for rats treated both pre- and postnatally, the mRNAs for nNOS, COX-2 and TGF- β were significantly depressed. Overall, transient prenatal exposure to Mn attenuated the response of several immune-related and neurotrophic genes to subsequent Mn exposures.

Fig. 4. Transcript levels of gene GFAP determined by quantitation of autoradiograms of Northern blots. Values were normalized to actin mRNA. Values represent the mean $\pm$ S.E. of three independent determinations of 3–5 animals from each group. (*) Mn-treated animals of this group differ ($p < 0.05$) from animals of other Mn-treated groups and those of the control group.

Fig. 5. Transcript levels of gene TGF- β determined by quantitation of autoradiograms of Northern blots. Values were normalized to actin mRNA. Values represent the mean $\pm$ S.E. of three independent determinations of 3–5 animals from each group. (*) Mn-treated animals of this group differ ($p < 0.05$) from animals of other Mn-treated groups and those of the control group.

To determine the effect of the exposures on Mn concentrations in the brain, neutron activation analysis was performed. Although levels of Mn were slightly higher among animals that were administered Mn as compared to those of the control group, these differences were not statistically significant. Levels of Mn in ppm in the control brains were 1.11 ± 0.26 while corresponding levels following pre- and post-natal exposure were 1.20 ± 0.01 and 1.30 ± 0.20 , respectively.

4. Discussion

Most studies involving Mn neurotoxicity in animals have focused on behavioral and biochemical endpoints following exposure to manganese in order to identify changes induced by an environmental exposure. A number of cellular processes, most notably neurotransmitter synthesis and catabolism, have been related to Mn-induced neurodegeneration (Donaldson et al., 1984; Graham, 1984; Mena et al., 1967). However, few in vivo studies have explored the initial molecular events that occur in response to excessive Mn exposure (Dobson et al., 2003; Dorman et al., 2004b; Erikson et al., 2004; Erikson et al., 2005). Thus, our consideration of changes in gene expression induced by inhalation exposure to Mn remains a relatively novel undertaking.

4.1. Amyloid precursor protein

The APP gene is constitutively expressed in neurons, and is inducible in glia (Banati et al., 1993; Beyreuther et al., 1996). The holoprotein is present normally as a molecule resembling a type I transmembrane receptor and metal-binding protein (Bush et al., 1994; Multhaup et al., 1996). The recent finding of distinct and specific binding sites for divalent metals in the 5'-untranslated region of the APP transcript suggests a regulatory

role for metals. The γ -secretase-cleaved C-terminal fragment has been associated with oxidant conditions, as well as inflammation (Rogers et al., 2002).

Rats exposed to Mn in adulthood had lower expression levels of this gene in the brain, whereas APP mRNA was not modulated in a significant manner in rats administered Mn during gestation. This suggests that Mn may be interfering with the normal transcriptional control of this gene by Fe.

4.2. Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) is one of two isoforms of an enzyme that catalyzes the conversion of arachidonic acid to prostanooids. This isoform is mitogen-inducible in the nervous system, whereas COX-1 gene is constitutively expressed (O'Banion, 1999). In rat brain, COX-2 mRNA is induced in neurons by synaptic activity and depressed by glucocorticoids (Adams et al., 1996; Kaufmann et al., 1996). It is rapidly elevated in response to inflammatory stimuli such as IL-1 β and lipopolysaccharide (LPS). COX-2 may also mediate cellular differentiation and cell survival. Activated glia may further induce the expression of the gene in neurons through the release of cytokines. In the brains of rats exposed to Mn by inhalation during adulthood, the expression of the gene encoding for COX-2 was significantly depressed, whereas levels of expression were non-significantly decreased in rats of both groups prenatally exposed to Mn. This pattern of COX-2 gene expression resembled that of other inflammation-related genes in this study.

4.3. Nitric oxide synthase

The exact role of the NOS family of enzymes in the stress response remains controversial. Roles proposed for nitric oxide range from intercellular signaling and necrotic killing, to apoptotic regulation and tissue remodeling (Droge, 2002). The gene encoding for nNOS, the predominant isoform found in neuronal tissue, was downregulated in rats administered Mn by inhalation during adulthood. In contrast, transcriptional activity of iNOS, whose expression is stimulated by immunological components such as cytokines and lipopolysaccharides (Droge, 2002), was not present at detectable levels in any of the four groups of animals, implying the absence of an acute inflammatory response. The selective decrease in nNOS levels suggests that the normal production of nitric oxide was undermined, and signaling cascades that maintain redox homeostasis, were impaired.

4.4. Glial fibrillary acidic protein

Mn-induced neurotoxicity may be reflected by functional changes in astrocytes, including the production of nitric oxide and the alterations of the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase (GADPH) (Normandin and Hazell, 2002). Because these cells have the capacity to sequester metals, scavenge free radicals, and modulate inflammatory/immune responses, they are vital to the defense system of the

brain against oxidative stress. Neuronal injuries induce secondary hypertrophy of astrocytes, and frequently enhance the expression of glial fibrillary acidic protein (GFAP) (O'Callaghan et al., 1995). Less commonly reported, are decreased levels of GFAP. Examples of this include congenital and acquired hyperammonemia (Albrecht et al., 1996), astrocytic infection by herpes virus in mice (Itoyama et al., 1991), and intracerebral injection of the astrocytic toxin α -aminoadipic acid in rats (Khurgel et al., 1996). Manganese decreased the protein expression of GFAP in cultured astrocytes (Chen and Liao, 2002). This is paralleled by our finding that expression of the gene is depressed in animals exposed to Mn in adulthood but not in those gestationally exposed.

4.5. Cytokines

The transcription levels of the genes encoding for IL-1 β and IL-6, two potent cytokines that promote inflammation, were undetectable in all experimental groups including control, and found to be unaltered by all exposures to Mn, respectively, again reflecting absence of inflammation. However, the expression of TGF- β , which protects neurons by downregulating the responses of glial cells to disease in the absence of inflammation (Vitkovic et al., 2001), was altered by the various Mn exposure parameters utilized in the study. The mRNA level of TGF- β was significantly lower following inhalation exposure to Mn during adulthood, whereas this depression was relieved by prior exposure to Mn during gestation. These changes suggest a compromised immune response.

4.6. Manganese levels in brain

Because the measurements of mRNA and Mn levels were conducted on whole rat brains, more localized differences in Mn levels among the experimental groups may have been diluted by the global scale of the assays. That is, the tendency of Mn to accumulate in specific regions, such as the globus pallidus, may have been obscured, and the impact of subchronic exposures to Mn may be more pronounced than the minor changes found here.

Collectively, these findings suggest that the genetic response to exposures to Mn differs markedly with the stage of development. Characterization of the transcriptional changes and their precise role in the stress response remains limited, since the exact functions of these genes remain to be determined. The expression patterns of genes encoding immune components and neurotrophic factors APP, COX-2, GFAP, nNOS, and TGF- β were modulated only in brains of rats exposed to Mn as adults, and this was in a downward direction. While excess levels of these factors can have adverse effects, their activity is associated with stress responses are a vital component of cellular defense mechanisms and their chronic down regulation may compromise such protective processes. This study reports no evidence of harmful effects of Mn inhalation. The adaptive responses that occur following environmental changes, may precede or prevent evidence of tissue injury.

Transient prenatal exposure to manganese attenuated this effect to a limited extent in animals subsequently exposed to Mn as adults. Thus, exposure to Mn during gestation may activate cellular defenses with potentially protective effects that are sustained into adulthood. This illustrates the critical nature of the stage of development in determining the nature of the response to Mn. Transcriptional responses to elevated Mn levels during development promote the establishment of adaptive cerebral mechanisms that may counter subsequent potentially deleterious conditions. In the event that the exposure occurs at a later stage of maturation, cellular adaptations elicited by Mn may be less successful in maintaining neural homeostasis.

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