7-2-2008

Subchronic Inhalation of soluable manganese induces expression of hypoxia-associated angiogenic genes in adult mouse lungs

Melanie Falgout

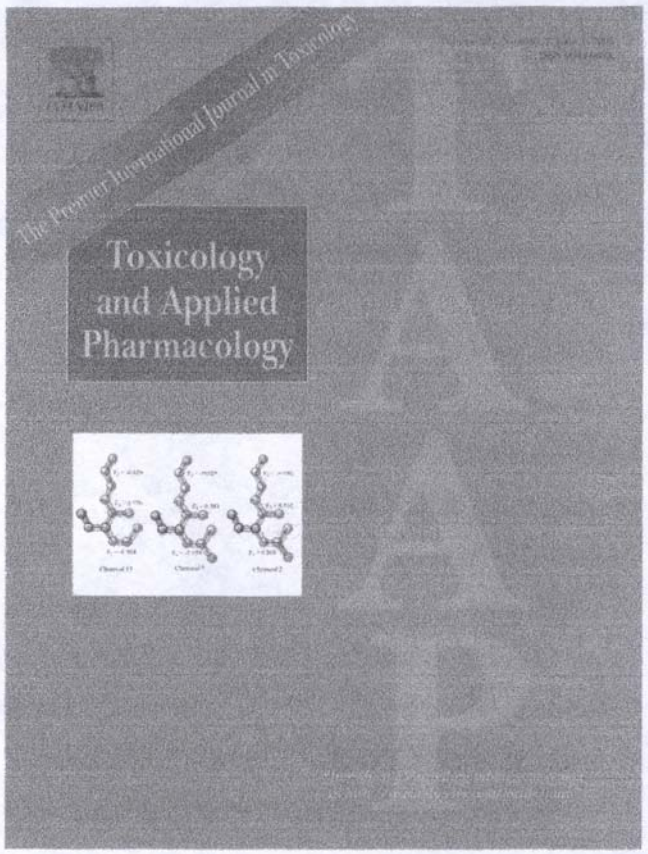
Sebastian Bredow

Thomas March

Christin Yingling

Stephen Malkoski

See next page for additional authors



Available online at www.sciencedirect.com
ScienceDirect
Toxicology and Applied Pharmacology 221 (2007) 148–157
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Subchronic inhalation of soluble manganese induces expression of hypoxia-associated angiogenic genes in adult mouse lungs

Sebastian Bredow [a,*], Melanie M. Falgout [a], Thomas H. March [a], Christin M. Yingling [a], Stephen P. Malkoski [a], James Aden [a], Edward J. Bedrick [b], Johnnye L. Lewis [c], Kevin K. Divine [a]

[a] *Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, USA*
[b] *Department of Math and Statistics, University of New Mexico, Albuquerque, NM 87131, USA*
[c] *Community Environmental Health Program, College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA*

Received 21 December 2006; revised 1 March 2007; accepted 1 March 2007
Available online 23 March 2007

Abstract

Although the lung constitutes the major exposure route for airborne manganese (Mn), little is known about the potential pulmonary effects and the underlying molecular mechanisms. Transition metals can mimic a hypoxia-like response, activating the hypoxia inducible factor-1 (HIF-1) transcription factor family. Through binding to the hypoxia-response element (HRE), these factors regulate expression of many genes, including vascular endothelial growth factor (VEGF). Increases in VEGF, an important biomarker of angiogenesis, have been linked to respiratory diseases, including pulmonary hypertension. The objective of this study was to evaluate pulmonary hypoxia-associated angiogenic gene expression in response to exposure of soluble Mn(II) and to assess the genes' role as intermediaries of potential pulmonary Mn toxicity. *In vitro*, 0.25 mM Mn (II) altered morphology and slowed the growth of human pulmonary epithelial cell lines. Acute doses between 0.05 and 1 mM stimulated VEGF promoter activity up to 3.7-fold in transient transfection assays. Deletion of the HRE within the promoter had no effect on Mn(II)-induced VEGF expression but decreased cobalt [Co(II)]-induced activity 2-fold, suggesting that HIF-1 may not be involved in Mn(II)-induced VEGF gene transcription. Nose-only inhalation to 2 mg Mn(II)/m^3 for 5 days at 6 h/day produced no significant pulmonary inflammation but induced a 2-fold increase in pulmonary VEGF mRNA levels in adult mice and significantly altered expression of genes associated with murine angiogenesis. These findings suggest that even short-term exposures to soluble, occupationally relevant Mn(II) concentrations may alter pulmonary gene expression in pathways that ultimately could affect the lungs' susceptibility to respiratory disease.

Keywords: Transition metals; Manganese; Inhalation; Hypoxia; VEGF; Angiogenesis

Introduction

Manganese (Mn) is widely distributed in soil, water, and the respirable particulate matter of air (Roth and Garrick, 2003). It is an essential trace element and required for normal mammalian physiological functions (Pine et al., 2005). When inhaled in excess, however, it produces serious health problems, predominantly in the central nervous system (CNS). Industrial processes are the main source for environmental exposure, and although the Occupational Safety and Health Administration (OSHA) regulations limit airborne Mn concentrations to 5 mg/m^3 air, concerns that even submicrogram concentrations could increase the risk of adverse health effects have frequently been discussed (Barceloux, 1999; Elsner and Spangler, 2005; Finley, 2004). For example, Mn is part of methylcyclopentadienyl manganese tricarbonyl (MMT), an additive in unleaded automotive gasoline (Frumkin and Solomon, 1997), and is primarily emitted in the soluble Mn(II)-sulfate and -phosphate forms after combustion (Molders et al., 2001). Experiments in adult rats have shown that following inhalation, elevated concentrations of these forms of the metal can be detected in brain and lung (Dorman et al., 2001; Salehi et al., 2003).

* Corresponding author. Fax: +1 505 348 8567.
E-mail address: sbredow@LRRI.org (S. Bredow).

0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2007.03.010

The lungs represent a potential first target for the metal's toxicity, although it is generally believed that the risk of lung disease from environmental Mn exposure is low (Barceloux, 1999; Oberdörster and Cherian, 1988). Soluble Mn(II) forms deposited in the lower airways of adult rats are quickly absorbed. Following intratracheal administration, their pulmonary clearance half-time is less than 0.5 day, with no effects on lung pathology (Vitarella et al., 2000). In rhesus monkeys inhalation for at least 15 days produced inflammatory changes in small airways, but the changes were absent 45 days post-exposure suggesting that Mn(II)-induced lesions are reversible after exposure termination (Dorman et al., 2005). Molecular *in vitro* studies, however, have demonstrated that exposures to soluble chloride forms of transition metals, specifically cobalt [Co(II)] and nickel [Ni(II)], induce a cellular response similar to the one caused by hypoxia (Maxwell and Salnikow, 2004).

Low-oxygen conditions play a pivotal role in physiological processes. They are also involved in the development of several pathological conditions, including stroke, cardiovascular disease, and cancer (Giaccia et al., 2003). Hypoxic conditions activate members of the hypoxia-inducible factor (HIF) transcription factor family and increase the expression of a number of genes, including those involved in angiogenesis (Maxwell and Salnikow, 2004; Wang et al., 1995). HIFs bind DNA as αβ-heterodimers at the hypoxia-response element (HRE), which is found within the promoters of target genes (Maxwell and Salnikow, 2004; Wang et al., 1995).

HIF-1 upregulates vascular endothelial growth factor (VEGF), one of the most important regulators of angiogenesis (Höpfl et al., 2004). VEGF exerts its mitogenic effects dose-dependently via binding to an endothelial-specific tyrosine kinase receptor, KDR/flk-1 (or VEGFR-2; Ferrara et al., 2003). The receptor-mediated response leads to proliferation, migration, and formation of new capillaries by the usually quiescent vascular endothelial cells (Ferrara et al., 2003). Under hypoxic conditions the response to elevated VEGF levels is mediated by a second receptor, fms-like tyrosine kinase-1 (flt-1/VEGFR-1) (Kaban and Herbst, 2002; Pugh and Ratcliffe, 2003). Elevated VEGF levels are associated with the pathogenesis of many diseases, including arthritis, macular degeneration, and cancer (Carmeliet, 2003).

Normal lungs, one of the most vascularized tissues, express high levels of VEGF (Marti and Risau, 1998), the function of which especially in the adult tissue is subject to ongoing investigations. VEGF is important for pulmonary vascular homeostasis and may play a role in maintaining the integrity of the air-blood barrier (Galambos et al., 2002). In mice, three major VEGF isoforms have been identified (Galambos et al., 2002). $VEGF_{188}$ is considered to be the physiologically relevant isoform. Its levels increase during development and remain high in the adult lungs while those for $VEGF_{120}$ and $VEGF_{164}$ increase only slightly and remain low (Galambos et al., 2002). However, $VEGF_{164}$ is associated with pulmonary disease, and overexpression of this isoform increases mortality in neonatal mice and causes hemorrhage, alveolar remodeling, and inflammation in surviving animals (Le Cras et al., 2004). In humans elevated VEGF levels are associated with the pathogenesis of some of the most common respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary hypertension, and lung cancer (Papaioannou et al., 2006).

Metals such as Co, Ni, and Mn are used in various industrial applications. Concerns involving potential adverse health effects from even sub-microgram metal exposures have increased, and understanding the underlying mechanisms of metal-induced toxicity has become an important issue. All three metals are known to induce VEGF expression *in vitro* (Gleadle et al., 1995; Maxwell and Salnikow, 2004). Both Co and Ni increase VEGF levels via a hypoxia-associated signaling cascade, with HIF-1 being implicated in mediating these effects (Maxwell and Salnikow, 2004; Vengellur and LaPres, 2004). Although Mn is similar to Co and Ni in its atomic structure, its effects on the hypoxia-associated pathway have been investigated in less detail. The objective of this exploratory study was, therefore, to determine whether inhaled soluble Mn(II) affects the expression of hypoxia-associated angiogenic genes in the adult mouse lung, and assess if such changes could mediate potential pulmonary Mn toxicity.

Methods

Cell culture. The human lung cancer-derived epithelial cell lines A549 and Calu-3 were used in these studies. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C in 100-mm culture dishes (USA Scientific; Ocala, FL) using RPMI medium (Invitrogen; Carlsbad, CA) supplemented with 10% (v/v) Fetal Bovine Serum (HyClone; Logan, UT), 100 U/ml penicillin, and 10 μg/ml streptomycin (all from Invitrogen). Cell stocks were subcultured when the cells covered the surface of the entire dish.

VEGF promoter constructs. The *SacI/NheI* fragment (−1175 to +52; restriction endonucleases and modifying enzymes were obtained from New England Biolabs; Beverly, MA) of the full-length VEGF promoter (Mukhopadhyay et al., 1995) was subcloned into the firefly luciferase reporter vector pGL2-Basic (Promega; Madison, WI), yielding expression plasmid p1.2. Promoter deletions with or without the hypoxia-response element (HRE; −986 to −922; Kimura et al., 2000) were created as follows. Plasmid p1.2 was digested with *Sac*I and *Pvu*II (−1167/−1014). The protruding 3′ termini were digested with the Klenow fragment of *E. coli* DNA polymerase I, and relegation yielded construct p1.01 (*−1014* to +52) that contained the HRE. Deletion of the HRE was achieved through PCR using primers that bind downstream of the HRE. The forward primer (−901 to −880) contained a *Sac*I restriction site (represented by lower-case letters: 5'-GCgagctcGCTCCACAAACTTGGTGCCAA-3'); GLprimer2 (Promega) was used as the reverse primer. The amplified fragment was *SacI/NheI* digested and ligated into *SacI/NheI*-digested pGL2-Basic, yielding construct p0.9 (−901 to +52). All constructs were verified by DNA sequencing.

Transient transfection assays. Cells (5×10^3 per well) were plated ~24 h before transfection. FuGENE 6 (Roche Diagnostics; Indianapolis, IN) was used as the transfection reagent at FuGENE to DNA amounts of 2:1. Two-hundred nanograms of promoter construct was co-transfected with 0.1 μg of *Renilla* luciferase-expressing reporter vector pRL-TK (Promega), which served as a control for well-to-well variation and transfection efficiency. Thirty-two hours later, cells were washed extensively and serum-starved for ~19 h. Cells were subsequently incubated for 20 h with complete medium containing either solvent or $MnCl_2$ (in tetrahydrate form and ≥99% pure; Acros Organics; Morris Plains, NJ; or $CoCl_2$, in hexahydrate form, cell culture-tested; Sigma-Aldrich; St. Louis, MO) in concentrations ranging from 0.05 mM to 1 mM. Cells were then lyzed and promoter activity was analyzed in a Luminoskan Ascent luminometer (Thermo Electron; Milford, MA) using the Dual Luciferase® Reporter (DLR) Assay System (Promega). Experimentally obtained firefly luciferase activity was normalized to *Renilla* activity and expressed as "fold

induction" over that of non-exposed control cells. All transfections were performed in duplicate and repeated at least three times unless otherwise stated.

Cell morphology and growth rate assessments. To document changes in morphology during growth in manganese-containing medium, 0.5 ml of cell suspension containing 2×10^4 cells was seeded in each well of a 24-well plate (USA Scientific; Ocala, FL). Mn(II) in the soluble chloride form was added immediately afterwards from 2.75 mM stocks, yielding a final concentration of 0.25 mM; controls received the appropriate amount (50 µl) of solvent (HEPES-buffered saline [HBS] pH 7.4). Cells were photographed every 24 h using an Olympus CK2 inverted microscope at 50× magnification. Mn(II)-containing medium, or medium containing HBS only, was changed every 24 h. When control cells became confluent (usually within 48 h), both control and Mn(II)-exposed cells were washed twice with phosphate-buffered saline, pH 7.2, and then incubated with 0.1 ml 0.25% trypsin (Invitrogen) until they detached. Cells were washed off in 0.5 ml medium, and 0.3 ml of cell suspension was mixed with 0.2 ml of medium and seeded in each well of a 24-well plate.

To obtain direct cell counts, 5×10^3 control or Mn-exposed cells in duplicate were grown for 1, 3, and 5 days as described above. Total cell counts for treated and non-treated cells per well were manually determined at these time points using a hemocytometer.

Animals. Twelve-week-old female FVB/N mice were purchased from Taconic (Germantown, NY). They were housed at 23 °C under pathogen-free conditions on a 12-h light/dark cycle for approximately 2 weeks prior to experiments in the institutional American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility with food and water available *ad libitum*. All experiments involving the animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the Lovelace Respiratory Research Institute (LRRI) and conducted in compliance with the regulations of the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Inhalation exposures. Mice were randomly assigned to 2 groups ($n=3$) and concurrently exposed to either clean air (control) or soluble $MnCl_2$-aerosol as described previously (Lewis et al., 2005). In brief, the aerosol was generated from an aqueous $MnCl_2$-solution using a RETEC nebulizer. The aerosol contained 2.0±0.36 mg Mn/m^3 air with an average particle size of 1.98±0.12 µm. Nose-only inhalation to the aerosol was carried out at a flow rate of 20 L/min for 6 h/day for 5 consecutive days. Immediately after completing the last exposure on day 5, animals were sacrificed by exsanguination followed by saline perfusion. The lungs were cut in half; one half was snap-frozen in liquid N_2 and stored at −80 °C for future RNA analyses while the other half was prepared for histopathology.

Histopathology. Following tracheal cannulation, lung halves were inflated with 10% neutral-buffered formalin. The tracheas were ligated and the lungs immersed in formalin for at least 24 h. The lungs were trimmed, and tissue blocks were processed and embedded in paraffin. Sections from the blocks were stained with hematoxylin and eosin for histopathologic evaluation.

Semiquantitative RT-PCR. Total RNA from a randomly selected lung lobe per animal (total of 3 per control and Mn(II)-exposed group) was extracted using TRI Reagent (Sigma-Aldrich) as previously described (Bredow et al., 2000). One microgram of RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Each RNA sample was reverse transcribed twice, and each cDNA sample was amplified in duplicate. Conditions for PCR reactions to remain in the linear range during amplification, using 2 µl of a 7-fold cDNA dilution, have been described previously (Bredow et al., 2000). The primer pairs used are listed in Table 1. The PCR products (10 µl) were separated on 2% agarose gels and visualized under UV illumination. Densitometric analyses were performed using a FluorS MultiImager with Quantity One 4.5.0 software (both from Bio-Rad Laboratories). Values per gene product for all control and exposed animals were normalized to those of $VEGF_{120}$, the expression of which remains stable in mouse lung (Galambos et al., 2002), averaged, and then expressed as "fold induction" over the data obtained from the non-exposed control animals.

Genomic microarray analyses. Different lung lobes from the ones used in the RT-PCR study were used in this experiment. Total RNA was extracted using an RNeasy Mini Kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. The Keck-UNM Genomics Resource facility (KUGR; http://hsc.unm.edu/som/micro/Genomics) at the University of New Mexico in Albuquerque, NM, determined quantity and quality of all 6 RNA samples, performed the cDNA and cRNA syntheses, and hybridized the 6 gene chips (Mouse Genome 430A 2.0 array representing approximately 14,000 well-characterized mouse genes) according to the DNA microarray manufacturer's instructions (Affymetrix; Santa Clara, CA). The hybridized arrays were washed and stained, using the Affymetrix Fluidics Station 450, and then scanned by the Agilent GeneChip Scanner 3000 (Agilent Technologies; Palo Alto, CA). The scanned output files were averaged with Affymetrix Microarray Suite 5.0 software, and further data analysis was conducted at LRRI using GeneSpring 7.2 software (Silicon Genetics; Redwood City, CA) as described (Currie et al., 2005). In brief, the array values were first normalized by setting all values ≤0 to 0.01 and then dividing the individual gene values by the chip median. Further analysis was restricted to gene values listed as present or marginal (Affymetrix flags) in at least 1 of the 3 samples of each group. To detect significant changes in expression levels, parametric *t*-tests were first conducted on the log values of the 2 exposure groups, followed by non-parametric Wilcoxon Mann–Whitney tests to corroborate robustness of the data. For both tests, changes in gene expression were deemed significant with a p-value ≤0.05.

Statistics. Differences between control and Mn-exposed groups in *in vitro*-experiments, with the exception of the dose–response experiments, were analyzed using Student's unpaired *t*-tests (two-tailed) with the significance set at $p<0.05$. Dose–response experiments shown in Fig. 1 were analyzed by one-way ANOVA followed by Dunnett's post-test; the linear trend between column means was analyzed at the same time.

Results

Effect of soluble Mn(II) on VEGF promoter activity in pulmonary cells

Various Co(II)- and Ni(II)-chloride concentrations have been used *in vitro* to increase VEGF mRNA expression at the transcriptional level (Gleadle et al., 1995; Maxwell and Salnikow, 2004; Pham et al., 2002). We performed transient transfection assays in human lung epithelial A549 and Calu-3 cells to study the effects of different Mn(II)-chloride concentrations on VEGF promoter activity. Cells transfected with an expression plasmid, in which the wild-type (wt) VEGF promoter controls firefly luciferase gene expression, were exposed to the soluble salt for 20 h (final concentration ranging from 0.05 to 1 mM). With the exception of the lowest concentration in both cell lines, and 0.1 mM in A549 cells, the exposure significantly induced promoter activity between 1.9-fold (Calu-3 at 0.1 mM) and 3.7-fold (Calu-3 at 1 mM) (Fig. 1; $p<0.05$). This induction varied among the cell lines, with A549 cells displaying lower activity (between 2-fold at 0.25 mM and 2.4-fold at 1 mM) than Calu-3 cells (between 1.9-fold at 0.1 mM and 3.7-fold at 1 mM).

Effect of the HRE on Mn(II)-induced VEGF promoter activity

A549 cells constitutively express HIF-1α (Pham et al., 2002), and exposing the cells to Co(II) further increases the

Table 1
RT-PCR primer

Gene/protein	Forward	Reverse	Product size
VEGF	CCATGAACTTTCTGCTGTCTT	TCACCGCCTCGGCTTGTCACAT	$VEGF_{188}$: 647 bp $VEGF_{164}$: 575 bp $VEGF_{120}$: 443 bp
Endoglin	CAATGCCAGCATTGTCACCTCC	AGAGGCTGTCCATGTCGATGCA	428 bp

levels of the subunit (Kimura et al., 2000; Li et al., 2006). This ultimately results in increased levels of HIF-αβ heterodimers, which cause enhanced VEGF expression through binding to the HRE found within the gene's promoter (Kimura et al., 2000; Li et al., 2006). To determine whether Mn(II)-induced VEGF promoter activity is similarly controlled, transient transfections were performed using VEGF reporter constructs with or without functional HIF-1 binding sites (Fig. 2). In comparison to non-exposed control cells, exposure to both metals increased promoter activity of expression plasmids containing the HRE several-fold (Co: 4.9-fold; Mn: 2.6-fold; $p < 0.0001$). Deletion of the HRE affected Co(II)-induced promoter activity (~2-fold decrease to 3.3-fold; $p = 0.003$) but not the one induced by Mn(II) (2.4-fold increase; $p = 0.63$), indicating that HIF-1 may not be obligatory for Mn(II)-induced VEGF gene transcription.

Effect of Mn(II) on pulmonary cell morphology and growth

The exposure concentration chosen to study the *in vitro* effects of soluble Mn(II) on pulmonary cells was based on previous cell survival data, which had demonstrated that concentrations ≤0.2 mM of elemental Mn were cytotoxic in normal human lung epithelial cells within 48 h (Pascal and Tessier, 2004). We did not test higher concentrations, but our data indicate that human lung cancer-derived A549 and Calu-3 cell lines were viable in medium with 0.25 mM Mn(II). However, this concentration affected the morphology of both cell lines. In comparison to control cells, which adopted the typical cobblestone appearance of epithelial cells, exposed cells adopted a spindle-like, elongated shape (Fig. 3A). The cells further maintained their altered appearance throughout the exposure. Similar to control cells, exposed cells could be passaged (Fig. 3B) and were grown in Mn(II)-containing medium for 2 weeks before exposures were terminated.

The presence of Mn(II) in medium, however, did significantly affect the growth of both cell lines (Fig. 4; $p \leq 0.0001$). In comparison to non-exposed A549 cells, a 65% reduction in cell number (32,250 control vs. 11,250 exposed cells per microliter) was found after 3 days in 0.25 mM $MnCl_2$-containing medium; this factor increased to 79% after 5 days of continuous exposure (109,875 vs. 23,250 cells/μl). Calu-3 cells appeared more resilient to Mn, and a reduction of 52% (39,500 vs. 18,875 cells/microliter) and 70% (159,875 vs. 47,250 cells/μl) in cell number was seen after 3 and 5 days of exposure.

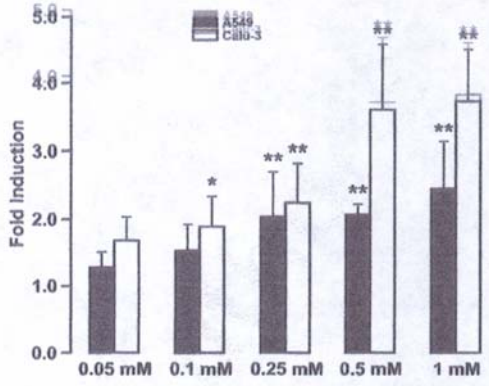


Fig. 1. VEGF promoter activity in response to different Mn(II) concentrations. Human lung cancer-derived epithelial cell lines (A549 black, and Calu-3 white columns) were transiently transfected with a plasmid in which the wild-type (wt) VEGF promoter controls expression of the reporter gene luciferase. $MnCl_2$ was added to the cells (final concentrations 0.05 mM to 1 mM) and promoter activity analyzed by Dual Luciferase® Reporter assay (Promega) after 20 h of exposure. Inductions are expressed over control, i.e., activity in non-exposed cells, which was set to 1. The mean±standard deviation (SD) of three independent transfections is shown. Mn(II) concentrations that significantly induced promoter activity in exposed over control cells are indicated (*$p < 0.05$; **$p < 0.01$). In both cell lines the test for linearity between column means revealed a significant linear trend ($p < 0.0001$).

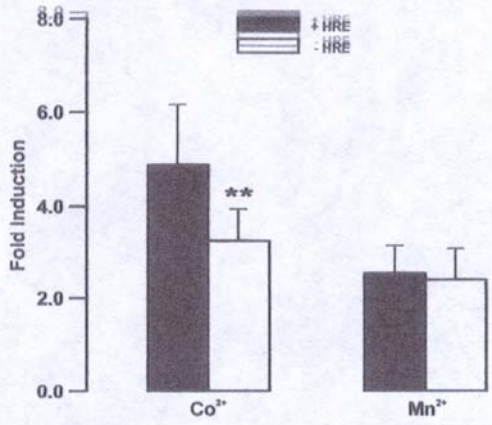


Fig. 2. Activity of VEGF promoter deletions in response to Co(II)- and Mn(II)-exposure. A549 cells were transiently transfected with either a plasmid containing a modified wt promoter (+HRE) or a plasmid in which the hypoxia-response element had been deleted (−HRE). Cells were exposed to 0.25 mM metal(II) chloride for 20 h followed by analysis of promoter activity as described. Inductions are expressed over control, i.e., activity in HBS-exposed cells, which was set as 1. The mean±SD of five independent transfections is shown. Absence of the HRE significantly affected Co-induced promoter activity (**$p < 0.01$), while stimulation with Mn was not affected.

Effect of inhaled Mn(II) on mouse lungs

To determine if the *in vitro* effects could be observed *in vivo*, we investigated the effects of $MnCl_2$-aerosol in adult mouse lungs after nose-only inhalation to an occupationally relevant concentration of 2 mg Mn/m^3 air. Following a 6 h/day exposure for 5 consecutive days, lung pathology remained unremarkable (Fig. 5A, control; 5B, Mn(II)-exposed). Other than a minor increase in the number of alveolar macrophages and rare neutrophils in the peribronchiolar interstitium, no significant compound-associated lesions were found in the lungs of exposed animals.

Effect of inhaled Mn(II) on VEGF expression in lung

Semiquantitative RT-PCR was conducted to determine VEGF mRNA levels in lungs of Mn(II)-exposed mice. The primer pairs used and the expected product lengths are described in Table 1. All three murine VEGF isoforms were detected in both unexposed and Mn(II)-exposed lungs (Fig. 6A, upper panel). Mn(II) affected expression levels of the housekeeping gene β-actin, intended for normalization of the PCR data (not shown). However, densitometric evaluation of the individual PCR products revealed that $VEGF_{120}$ mRNA levels were the least affected by Mn(II), and all values were, therefore, normalized to the corresponding $VEGF_{120}$ values. Results, expressed as "fold induction" of normalized values from exposed lungs over those from control animals reveal that Mn(II) only increased $VEGF_{188}$ levels (Fig. 6B, ~1.5-fold; $p=0.101$). We also determined mRNA levels for the proliferation-associated endothelial cell surface receptor endoglin (CD105) and detected a 1.8-fold increase in Mn(II)-exposed lungs (Fig. 6A, lower panel, and Fig. 6B; $p=0.0007$) (Kumar et al., 1996).

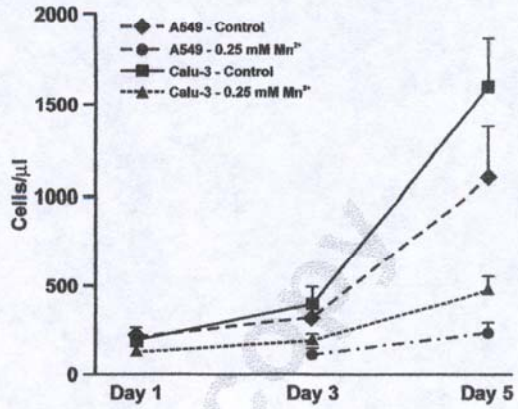


Fig. 4. Growth of A549 and Calu-3 cells in response to Mn(II) exposure. Cells (5000) were seeded in duplicate and maintained in regular (control) or Mn(II)-containing medium (0.25 mM Mn^{2+}) for the time indicated, when they were trypsinized and their total cell number determined. The numbers, expressed as cells per microliter of medium, between control and Mn(II)-exposed cells varied significantly on days 3 and 5 ($p \leq 0.0001$). The cell number for Mn(II)-exposed A549 cells on day 1 could not be determined and there was no significant difference in cell number between control and Mn(II)-exposed Calu-3 cells on day 1 ($p=0.1925$).

Effect of inhaled Mn(II) on angiogenesis-associated gene expression in lung

A microarray study using RNA prepared from different lung lobes from the ones used in the RT-PCR study was performed to identify additional angiogenesis-associated genes for which the expression was affected by soluble Mn(II). A query of the NCBI

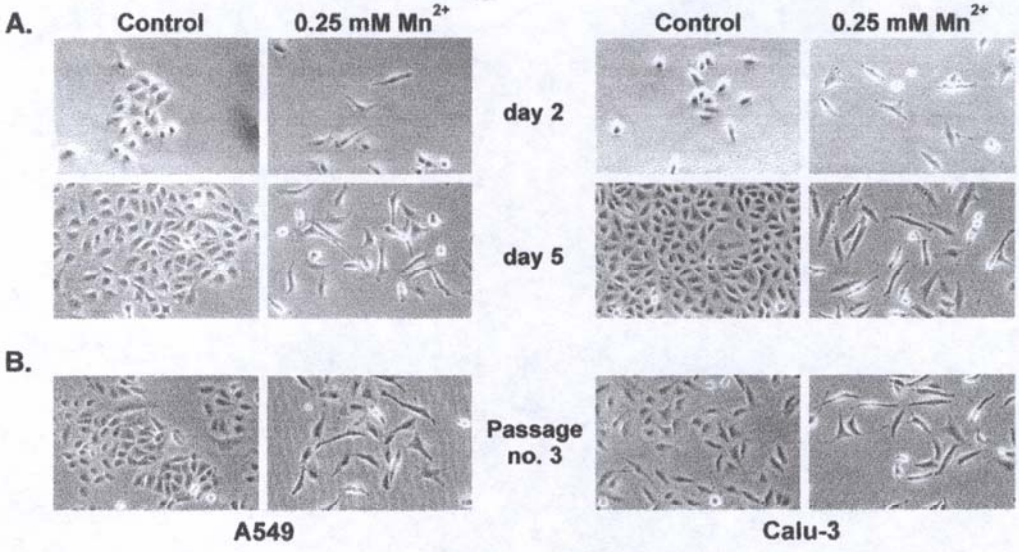


Fig. 3. Morphology of A549 and Calu-3 cells in response to Mn(II) exposure. Cells were grown in either regular (control) or Mn(II)-containing medium (0.25 mM Mn^{2+}). (A) Changes in cell morphology were observed 2 (top panels) and 5 days (lower panels) after continuous growth in 0.25 mM of $MnCl_2$-containing (right panels) but not control (left panels) medium for A549 (left 2 panels) and Calu-3 (right 2 panels) cells. (B) Morphology of A549 (left 2 panels) and Calu-3 (right 2 panels) cells in control (left panels) and Mn(II)-containing (right panels) medium after three cell passages. Upon confluency, all cells were treated with trypsin, washed off with medium, and re-seeded in new plates. Cells were photographed by phase contrast light microscopy at 50× magnification.

database using the search term "angiogenesis and mus musculus" yielded approximately 70 genes that affect biological processes in the vasculature of mice. Searching for those candidates within the obtained microarray gene list identified 27 angiogenesis-associated genes, corresponding to 38 active spots on the Mouse Genome 430A 2.0 array (Table 2). Overall, Mn appeared to induce and not repress gene expression. Only two genes, KLF5 and Tbx1, showed decreased expression, while expression levels of 25 genes were significantly increased ($p < 0.05$). Furthermore, the exposure induced more pro- than anti-angiogenic genes. Of the 27 candidates, 23 genes are associated with pro-angiogenic function while four genes – Robo4, thrombospondin-1 (Thbs1), Col18a1 (endostatin), and Rhob – are associated with anti-angiogenic function (Table 2).

Confirming the RT-PCR data, Mn(II) significantly affected *in vivo* expression of VEGF in mouse lungs (Table 2). Increased levels for $VEGF_{188}$ (1.9-fold; $p=0.028$) and the alternatively spliced murine isoform $VEGF_{115}$ (2.7-fold; $p=0.016$), the function of which remains unknown (Sugihara et al., 1998), were detected. No increases in the two other VEGF isoforms were measured. However, endoglin expression levels were also increased (~2-fold, 2 spots). Hypoxia causes increases of VEGF and its receptor VEGFR-1 via HIF-1 (Kaban and Herbst, 2002). Similarly, Mn(II) significantly increased expression levels of VEGFR-1 (≥2-fold, 2 spots). Expression levels for both HIFα-subunits, HIF-1α (≤1.4-fold; 2 spots) and HIF-2α (2.78-fold; $p=0.017$), also known as Epas 1 (endothelial PAS domain protein 1) (Höpfl et al., 2004), were further increased in response to Mn(II) exposure.

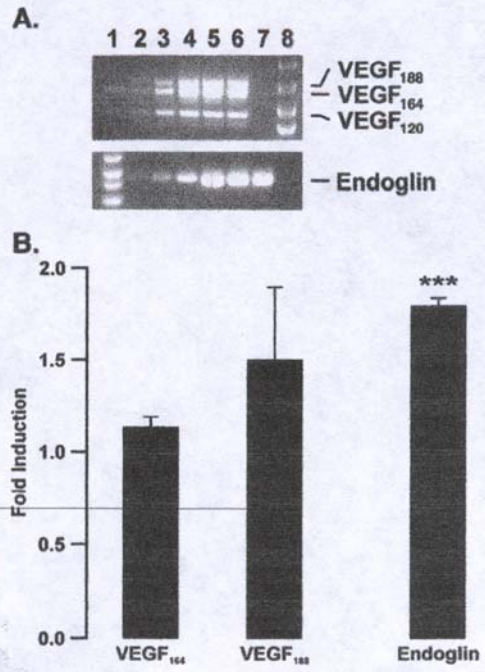


Fig. 6. Semiquantitative RT-PCR of Mn(II)-exposed mouse lungs. Mice were exposed to $MnCl_2$-aerosol as described. Total RNA was extracted from lungs and converted into cDNA. Amplifications of the VEGF isoforms and endoglin, respectively, are shown for 38 and 32 cycles; the expected fragment lengths are listed in Table 1. (A) Analysis of 10 µl of PCR product in 2% agarose gels. VEGF upper panel: lanes 1–3 non-exposed lungs, lanes 4–6 Mn(II)-exposed lungs, lane 7 PCR control (no DNA), lane 8 DNA Marker (1 kB Plus Ladder, Invitrogen). Endoglin lower panel: lane 1 DNA Marker, lanes 2–4 non-exposed lungs, lanes 5–7 Mn(II)-exposed lungs, lane 8 PCR control. (B) Analysis of band density. Values, normalized to $VEGF_{120}$ levels, are expressed as "fold induction" of mRNA levels detected in the three Mn-exposed mouse lungs over those detected in the three non-exposed tissues. Data are represented as mean±SD, and significant fold changes in Mn-exposed lungs are indicated (***$p < 0.001$).

Discussion

Previous *in vitro* exposures conducted under normoxic conditions have demonstrated that the soluble chloride forms of transition metals, specifically cobalt [Co(II)] and nickel [Ni(II)], mimic a cellular response similar to hypoxia (Maxwell and Salnikow, 2004). Hypoxic conditions alter the expression of genes involved in glycolysis, erythropoiesis, apoptosis, and

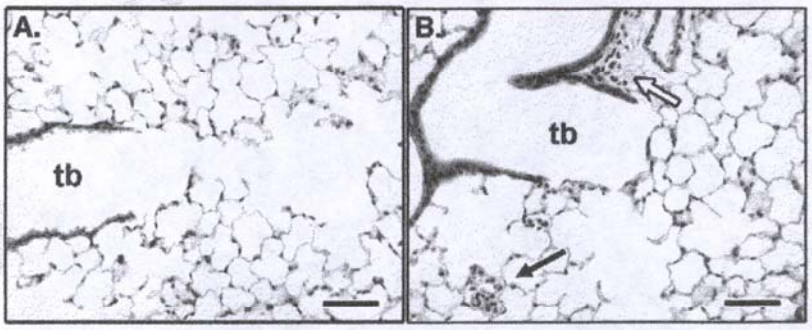


Fig. 5. Histopathology of murine lungs in response to Mn(II) exposure. Mice were exposed by nose-only inhalation to 2.0±0.36 mg Mn/m^3, generated from an aqueous $MnCl_2$-solution, for 6 h/day for 5 consecutive days. Following tracheal cannulation, lungs were fixed in formalin, and processed and embedded in paraffin. Sections were stained with hematoxylin and eosin. The centriacinar zone of pulmonary parenchyma in control (A) and Mn-exposed (B) mice is shown, with the terminal bronchioles (tb) indicated. These exposure conditions were associated with slight inflammation characterized by widely scattered alveolar exudates of mononuclear inflammatory cells and rare neutrophils along with alveolar macrophage hyperplasia (black arrow). Similar cells in small numbers were sometimes apparent in the interstitium around blood vessels and bronchioles (white arrow).

Table 2
Differentially regulated genes in Mn-exposed mouse lungs—angiogenesis

Gene Bank accession	Gene name	Gene symbol	Fold change[a,b]	*p*-value
a. Pro-angiogenic increased—growth factor/receptor				
NM_009505	Vascular endothelial growth factor A; 188 isoform	Vegfa	1.94	0.028
U50279	Vascular endothelial growth factor A; 115 isoform	Vegfa	2.70	0.016
NM_009506	Vascular endothelial growth factor C	Vegfc	4.05	0.0057
NM_010216	c-*fos* induced growth factor; VEGF-D	Figf	1.75	0.046
NM_010228	FMS-like tyrosine kinase 1; VEGFR-1	Flt1	1.96/ 3.35	0.018/ 0.033
D88690	FMS-like tyrosine kinase 1, soluble form	sFlt1	2.18	0.0047
NM_007932	Endoglin; CD105	Eng	1.81/ 2.39	0.0002/ 0.005
NM_010217	Connective tissue growth factor	Ctgf	1.78	0.026
NM_013749	Tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	4.22	0.049
BB133079	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	Edg1	3.98	0.0034
b. Pro-angiogenic increased—morphogenesis/remodeling				
NM_013690	Endothelial-specific receptor tyrosine kinase; Tie2	Tek	4.14	0.0028
NM_011587	Tyrosine kinase receptor 1	Tie1	2.93	0.02
NM_153423	WAS protein family, member 2	Wasf2	1.36	0.018
AK004739	Delta-like 4 (*Drosophila*)	Dll4	1.80/ 2.77	0.0019/ 0.033
NM_024226	Reticulon 4	Rtn4	1.53	0.045
NM_007554	Bone morphogenetic protein 4	Bmp4	1.84	0.031
NM_030711	Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	Arts1	1.26	0.035
BC024610	EGF-like domain 7; VE-statin	Egfl7	1.78/ 1.98	0.024/ 0.26
c. Pro-angiogenic increased – intracellular signaling				
BC012235	Mitogen activated protein kinase 14; p38α, p38MAPK	Mapk14	1.03	0.048
d. Pro-angiogenic increased – transcriptional regulation				
X95880	Hypoxia inducible factor 1, alpha subunit	Hif1a	1.14/ 1.39	0.032/ 0.037
U81983	Endothelial PAS domain protein 1; HIF-2α	Epas1	2.78	0.017
BC005686	ELK3, member of ETS oncogene family	Elk3	1.82/ 2.63	0.0045/ 0.021
U43884	Inhibitor of DNA binding 1; Idb1	Id1	2.48	0.0049
e. Pro-angiogenic decreased—transcriptional regulation				
AF326960	T-box 1	Tbx1	0.26	0.012
BC006646	Krüppel-like factor 5	Klf5	0.38/ 0.62	0.011/ 0.013
f. Anti-angiogenic increased—growth factor/receptor				
BC020129	Roundabout homolog 4 (*Drosophila*)	Robo4	1.97	0.0052
g. Anti-angiogenic increased—extracellular matrix				
AI385532	Thrombospondin 1	Thbs1	1.80/ 2.40	0.026/ 0.046
NM_009929	Procollagen, type XVIII, alpha 1; endostatin	Col18a1	1.72	0.042
h. Anti-angiogenic increased—intracellular signaling				
BC018275	ras homolog gene family, member B; Arhb	Rhob	1.96	0.0054

[a] The data represent fold change where $n=3$ arrays per treatment.
[b] A single array contains >22,600 probe sets representing transcripts and variants from over 14,000 well-characterized genes. Thus, the same gene can be detected with more than one probe.

angiogenesis (Höpfl et al., 2004; Vengellur and LaPres, 2004). Hypoxic conditions also influence the development of many pathological disease states (see Giaccia et al., 2003). The master regulators mediating the mammalian molecular response to hypoxia are members of the HIF-1 transcription factor family (Höpfl et al., 2004; Semenza, 2005). Co(II) and Ni(II) both stabilize HIF-1 (Maxwell and Salnikow, 2004), subsequently causing increases in hypoxia-associated gene expression. The activation of the hypoxia-inducible signaling cascade is believed to be a potential mechanism for their toxicity (Maxwell and Salnikow, 2004; Vengellur and LaPres, 2004). Mn(II) is structurally similar to Co(II) and Ni(II), but its effects on the hypoxia-inducible pathway have not been well characterized. Exposure to Mn(II) *in vitro* is arguably best known for inducing the expression of VEGF, an important biomarker of angiogenesis (Gleadle et al., 1995). Elevated pulmonary levels of VEGF are associated with the pathogenesis of diseases including asthma, COPD, pulmonary hypertension, and lung cancer (Papaioannou et al., 2006). To our knowledge this is the first study to demonstrate changes in hypoxia-associated angiogenic gene expression in pulmonary cells in response to Mn(II).

It has previously been demonstrated that exposures to concentrations ≤0.2 mM of elemental Mn are cytotoxic to normal human lung epithelial cells within 48 h (Pascal and Tessier, 2004). Our data demonstrate that *in vitro* exposures to 0.25 mM Mn(II) produced no visible toxic effects in human lung cancer-derived epithelial cells. The cells were viable in Mn(II)-containing medium for at least 2 weeks. However, the metal did induce changes in cell morphology and significantly slowed cell growth (Figs. 3 and 4). These *in vitro* data suggest that brief exposures of pulmonary cells to concentrations ≤0.25 mM of soluble Mn(II) are not cytotoxic. They are, however, sufficient to induce phenotypic changes. To our knowledge, significant

morphological changes of pulmonary cells in response to hypoxia or other transition metals have not been described.

Hypoxia appears to be the only known stressor that leads to increased VEGF expression in human lung epithelial cells (Boussat et al., 2000). In these cells the mechanism of hypoxia-induced VEGF upregulation is primarily transcriptionally controlled by HIF-1, resulting in a 3-fold increase in VEGF mRNA and a 2.5-fold increase in protein (Pham et al., 2002). Our data demonstrate that exposures to the soluble chloride form of Mn also increased VEGF promoter activity *in vitro*. The data from transient transfection assays further indicate that Mn (II)-induced VEGF expression is transcriptionally controlled. Mn(II) increased VEGF promoter activity in a concentration-dependent manner (Fig. 1). In the case of the Calu-3 cells, the level of stimulation ($\leq$3.7-fold) was similar to those induced by hypoxia or 0.25 mM $CoCl_2$ in rat alveolar epithelial cells (Pham et al., 2002). Mechanistically, however, the Mn(II)-induced upregulation appears different from the hypoxia- or Co(II)-induced VEGF activation. Deletion of the HRE, the HIF-1 binding site, from the VEGF promoter decreased Co(II)- but not Mn(II)-induced promoter activity, indicating that Mn(II)-induced transcriptional activity may be mediated independently of HIF-1 (Fig. 2). Future studies will determine which region of the VEGF promoter mediates Mn(II)-induced activity. Changes induced by Mn(II) may thus be similar but are not identical to the ones promoted by hypoxia or the other transition metals, making it likely that Mn(II) may affect only some but not all of the genes that are induced by hypoxia. The pulmonary response to Mn(II) may, therefore, be different from the one to hypoxia.

Hypoxic conditions increase transcription of VEGF and its receptor VEGFR-1 via HIF-1 (Höpfl et al., 2004; Kaban and Herbst, 2002). In the lungs of most species chronic hypoxia increases the medial thickness of pulmonary arterioles that, in concert with structural alterations of lung vessels (pulmonary vascular remodeling), causes pulmonary hypertension, a disease that ultimately leads to heart failure (Carmeliet, 2003; Christou et al., 1998). The data from our *in vivo* exposure indicate that inhaled soluble Mn(II) significantly increases the mRNA levels of VEGF, VEGFR-1, and the HIFα-subunits in the adult mouse lung.

HIF-1 mediates the toxicity of several metals such as Co, which in lung is associated with an increased risk for asthma and pneumonia (Li et al., 2006; Vengellur and LaPres, 2004). It is known that hypoxic conditions cause a rapid accumulation of HIF-1α without affecting the mRNA level (Yuan et al., 2003). Inhalation of Mn(II) produced a slight, albeit significant $\leq$1.4-fold increase in HIF-1α mRNA levels (Table 2). We did not measure HIF-1α protein levels and HIF-1 may be involved in mediating the response to Mn(II) *in vivo*. We further detected increased levels for another member of the HIF family, HIF-2α, the expression of which was induced nearly 3-fold in Mn(II)-exposed lungs. Unlike the ubiquitously expressed HIF-1α, HIF-2α is expressed in a tissue-specific manner (Semenza, 2005). The factor can also regulate a different set of genes, although some are involved in angiogenesis (Höpfl et al., 2004).

The lungs, as one of the most vascularized tissues in the body, express high levels of VEGF under normal physiological conditions (Marti and Risau, 1998). However, the role of VEGF in the adult lung is poorly understood, with contradictory evidence suggesting both protective and harmful roles (Papaioannou et al., 2006). It has been demonstrated that changes in its expression play a significant role in the development of some of the most common respiratory disorders such as asthma, COPD, and pulmonary hypertension (Papaioannou et al., 2006; Voelkel et al., 2006). VEGF is strongly expressed in the angioproliferative plexiform lesions in the lungs from patients with severe primary idiopathic and secondary forms of pulmonary hypertension (Voelkel et al., 2006). Although animal models mostly reflect milder forms of the disease, they allow investigating the role of a given growth factor in the initiation and progression of the disease. Results from these studies indicate that chronic hypoxia-induced VEGF is the likely mediator of pulmonary vascular remodeling (Papaioannou et al., 2006; Voelkel et al., 2006).

Mice express three VEGF isoforms of which, unlike in humans, the longest, $VEGF_{188}$, is considered to be the physiologically relevant one (Galambos et al., 2002). It is, however, the overexpression of the murine equivalent of the predominant human isoform $VEGF_{164}$ that causes vascular damage and alveolar remodeling in the adult mouse lung (Le Cras et al., 2004). It is believed that this isoform is part of the mechanism that mediates the early stages of acute lung injury (Kaner et al., 2000), while a potential role for $VEGF_{188}$ in disease has to our knowledge not been identified. We exposed adult mice for 5 days to an occupationally relevant Mn(II) concentration and, similar to that observed during acute hypoxia, detected a 2-fold increase in pulmonary VEGF mRNA levels (Christou et al., 1998; Pham et al., 2002). However, the increases were only detected in the longer but not the shorter disease-associated VEGF isoform (Table 2). Since it has been demonstrated that VEGF mRNA levels may not significantly increase during acute hypoxia but increase progressively after 1–3 weeks of hypoxia (Christou et al., 1998), it seems probable that the exposure conditions of this exploratory study were too short to induce significant expression of $VEGF_{164}$.

The exposure also affected the expression of other genes that are associated with pulmonary vasculature. We measured a $\geq$2-fold increase in VEGFR-1 and a 4-fold increase in the endothelial-specific receptor tyrosine kinase for angiopoietins, tie-2, in Mn(II)-exposed mouse lungs (Table 2). Hypoxic conditions lead to increases in VEGFR-1 via HIF-1 (Kaban and Herbst, 2002), while HIF-2α mediates tie-2 expression (Höpfl et al., 2004). Both molecules fulfill important functions during the maturation of blood vessels (Kaban and Herbst, 2002; Kearney et al., 2004). We further detected a 2-fold increase of the endothelial cell proliferation-associated marker endoglin (CD105; Table 2). Also induced by hypoxia, this receptor for the transforming growth factor (TGF)-β family is required for the formation of mature blood vessels (Duff et al., 2003). Endoglin is normally expressed in low levels but becomes strongly upregulated in the endothelium of tissues undergoing angiogenesis (Kumar et al., 1996).

However, other than the observed genotypic changes, phenotypic changes in lung were not observed, and pulmonary structures remained unaffected by this relatively short exposure.

Histopathologic findings did not indicate changes in pulmonary vasculature and revealed only minimal recruitment of inflammatory cells (Fig. 5). This is in agreement with a recent inhalation study by Dorman et al. (2005). These authors demonstrated that inhalation for > 1 week (6 h/day, 5 days/week for 13 weeks) caused minor inflammatory changes in small airways of rhesus monkeys in response to a different form of soluble Mn(II), manganese sulfate. In the absence of observable clinical signs, mild subacute bronchiolitis, alveolar duct inflammation, and proliferation of bronchus-associated lymphoid tissue were only detected after exposure to Mn(II) at 1.5 mg Mn/m^3 for ≥ 15 days. More importantly, these pathologic changes were reversible upon exposure cessation.

Growing evidence suggests that respirable matter can induce lung injury under certain exposure conditions (Dorman et al., 2005). However, it may take years before clinical symptoms become apparent (e.g., COPD, or lung cancer both resulting from cigarette smoking). Responses of the vasculature to induced genotypic changes are in particular slow (Pugh and Ratcliffe, 2003), potentially requiring weeks before phenotypical changes manifest (Dor et al., 2002; Kaner et al., 2000). Under these circumstances the relatively short exposure time potentially explains the lack of detectable pathological changes in response to Mn(II). Further, expression levels of only 27 of the at least 70 genes regulating vascular physiology were affected by the exposure. Phenotypic changes in the vasculature require altering a tightly coordinated cascade of temporal and spatial events (Carmeliet, 2003; Kaban and Herbst, 2002), and exposing animals to Mn(II) for 5 days appears insufficient to produce changes in vasculature. Both *in vitro* and *in vivo* data suggest that the pulmonary response to Mn(II) under these exposure conditions differs from those induced by hypoxia or other transition metals, potentially producing a different pulmonary phenotype. Given that pathological changes induced by subchronic exposures are also reversible (Dorman et al., 2005), it is likely that chronic long-term exposures will be required to determine adverse pulmonary effects of Mn(II).

In conclusion, this study demonstrates that a brief subchronic exposure to an occupationally relevant concentration of soluble Mn(II) produces genotypic changes in a pathway directly linked to respiratory diseases. However, further studies involving chronic exposures to inhaled Mn(II) are required to determine if the genotypic alterations produce phenotypic changes and pulmonary toxicity. Similar to neurotoxic Mn-associated problems, it is feasible that pulmonary Mn(II)-associated effects in humans become detectable only over time (Barceloux, 1999). In addition to the genes discussed, the exposure also affected expression levels of genes involved in other cellular pathways. Future studies, currently beyond the scope of this investigation, will determine if these genes and their pathways can mediate potentially harmful pulmonary health effects resulting from exposure to Mn(II).

Acknowledgments

We thank Dr. D. Mukhopadhyay (Mayo Clinic Cancer Center; Rochester, MN) for providing the VEGF plasmid that contains the full-length wt promoter (−2600 to +52), and Dr. G. Pickett and M. Morgan of UNM's KUGR facility for performing the microarray analyses. We are grateful to Dr. S. Belinksy for critically reading the manuscript, and S. Randock for help with the figures. This research was in part supported by DOD grant DAMD17-01-1-0297 (S. Bredow) and The New Mexico Center for Environmental Health Sciences NIEHS Grant P30-ES-012072 and NIEHS Grant 5R01-ES-010766-05 (J.L. Lewis). The sponsors of this research were not involved in the study design; collection, analysis, and interpretation of data; in the writing of the manuscript; or the decision to submit the paper for publication.

References

Barceloux, D.G., 1999. Manganese. Clin. Toxicol. 37, 293–307.

Boussat, S., Eddahibi, S., Coste, A., Fatacciolo, V., Gouge, M., Housset, B., Adnot, S., Maitre, B., 2000. Expression and regulation of vascular endothelial growth factor in human pulmonary epithelial cells. Am. J. Physiol. Cell Mol. Physiol. 279, L371–L378.

Bredow, S., Lewin, M., Hofmann, B., Marecos, E., Weissleder, R., 2000. Imaging of tumour neovasculature by targeting the TGF-β binding receptor endoglin. Eur. J. Cancer 36, 675–681.

Carmeliet, P., 2003. Angiogenesis in health and disease. Nat. Med. 9, 653–660.

Christou, H., Yoshida, A., Arthur, V., Morita, T., Kourembanas, S., 1998. Increased vascular endothelial factor production in the lungs of rats with hypoxia-induced pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 18, 768–776.

Currie, R.A., Bombail, V., Oliver, J.D., Moore, D.J., Lim, F.L., Gwilliam, V., Kimber, I., Chipman, K., Moggs, J.G., Orphanides, G., 2005. Gene ontology mapping as an unbiased method for identifying molecular pathways and processes affected by toxicant exposure: application to acute effects caused by the rodent non-genotoxic carcinogen diethylhexylphthalate. Toxicol. Sci. 86, 453–469.

Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G.I., Carmeliet, P., Goelman, G., Keshet, E., 2002. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J. 8, 1939–1947.

Dorman, D.C., Struve, M.F., James, R.A., Marshall, M.W., Parkinson, C.U., Wong, B.A., 2001. Influence of particle solubility on the delivery of inhaled manganese to the rat brain: manganese sulfate and manganese tetroxide pharmacokinetics following repeated (14-day) exposure. Toxicol. Appl. Pharmacol. 170, 79–87.

Dorman, D.C., Struve, M.F., Gross, E.A., Wong, B.A., Howroyd, P.C., 2005. Sub-chronic inhalation of high concentrations of manganese sulfate induces lower airway pathology in rhesus monkeys. Respir. Res. 6, 121–130.

Duff, S.E., Li, C., Garland, J.M., Kumar, S., 2003. CD105 is important for angiogenesis: evidence and potential applications. FASEB J. 17, 984–992.

Elsner, R.J.F., Spangler, J.G., 2005. Neurotoxicity of inhaled manganese: public health danger in the shower? Med. Hypotheses 65, 607–616.

Ferrara, N., Gerber, H.-P., LeCouter, J., 2003. The biology of VEGF and its receptors. Nat. Med. 9, 669–676.

Finley, J.W., 2004. Does environmental exposure to manganese pose a health risk to healthy adults? Nutr. Rev. 62, 148–153.

Frumkin, H., Solomon, G., 1997. Manganese in the U.S. gasoline supply. Am. J. Ind. Med. 31, 107–115.

Galambos, C., Ng, Y.-S., Ali, A., Noguchi, A., Lovejoy, S., D'Amore, P.A., DeMello, D.E., 2002. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. Am. J. Respir. Cell Mol. Biol. 27, 194–203.

Giaccia, A., Siim, B.G., Johnson, R.S., 2003. HIF-1 as a target for drug development. Nat. Rev. Drug Discov. 2, 803–811.

Gleadle, J.M., Ebert, B.L., Firth, J.D., Ratcliffe, P.J., 1995. Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. Am. J. Physiol. 268, C1362–C1368.

Höpfl, G., Ogunshola, O., Gassmann, M., 2004. HIFs and tumors—causes and consequences. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R608–R623.

Kaban, K., Herbst, R.S., 2002. Angiogenesis as a target for cancer therapy. Hematol. Oncol. Clin. N. Am. 16, 1125–1171.

Kaner, R.J., Ladetto, J.V., Singh, R., Fukuda, N., Matthay, M.A., Crystal, R.G., 2000. Lung overexpression of the vascular endothelial growth factor gene induces pulmonary edema. Am. J. Respir. Cell Mol. Biol. 22, 657–664.

Kearney, J.B., Kappas, N.C., Ellerstrom, C., DiPaola, F.W., Bautch, V.L., 2004. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. Blood 103, 4527–4535.

Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M., Esumi, H., 2000. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood 95, 189–197.

Kumar, P., Wang, J.M., Bernabeu, C., 1996. CD105 and angiogenesis. J. Pathol. 178, 363–366.

Le Cras, T.D., Spitzmiller, R.E., Albertine, K.H., Greenberg, J.M., Whitsett, J. A., Akeson, A.L., 2004. VEGF causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 287, L134–L142.

Lewis, J., Bench, G., Myers, O., Tinner, B., Staines, W., Barr, E., Divine, K.K., Barrington, W., Karlsson, J., 2005. Trigeminal uptake and clearance of inhaled manganese chloride in rats and mice. Neurotoxicology 26, 113–123.

Li, Q., Chen, H., Huang, X., Costa, M., 2006. Effects of 12 metal ions on iron regulatory protein 1 (IRP-1) and hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-regulated genes. Toxicol. Appl. Pharmacol. 213, 245–255.

Marti, H.H., Risau, W., 1998. Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. Proc. Natl. Acad. Sci. U. S. A. 95, 15809–15814.

Maxwell, P., Salnikow, K., 2004. HIF-1: an oxygen and metal responsive transcription factor. Cancer Biol. Ther. 3, 29–35.

Molders, N., Schilling, P.J., Wong, J., Roos, J.W., Smith, I.L., 2001. X-ray fluorescence mapping and micro-XANES spectroscopic characterization of exhaust particulates emitted from auto engines burning MMT-added gasoline. Environ. Sci. Technol. 35, 3122–3129.

Mukhopadhyay, D., Tsokias, L., Sukhatme, V.P., 1995. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res. 55, 6161–6165.

Oberdörster, G., Cherian, G., 1988. Manganese. In: Clarkson, T.W., Friberg, L., Nordberg, G.F., Sager, P.R. (Eds.), Biological Monitoring of Toxic Metals. Plenum Press, New York, pp. 283–301.

Papaioannou, A.I., Kostikas, K., Kollia, P., Gourgoulianis, K.I., 2006. Clinical implications for vascular endothelial growth factor in the lung: friend and for? Respir. Res. 7, 128–140.

Pascal, L.E., Tessier, D.M., 2004. Cytotoxicity of chromium and manganese to lung epithelial cells in vitro. Toxicol Lett. 147, 143–151.

Pham, I., Uchida, T., Planes, C., Ware, L.B., Kaner, R., Matthay, M.A., Clerici, C., 2002. Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. Am. J. Physiol. Lung Cell Mol. Physiol. 283, L1133–L1142.

Pine, M., Lee, B., Dearth, R., Hiney, J.K, Dees, W.L., 2005. Manganese acts to stimulate luteinizing hormone secretion: a potential influence on female pubertal development. Toxicol. Sci. 85, 880–885.

Pugh, C.W., Ratcliffe, R.J., 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. 9, 677–684.

Roth, J.A., Garrick, M.D., 2003. Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. Biochem Pharmacol. 66, 1–13.

Salchi, F., Krewski, D., Mergler, D., Normandin, L., Kennedy, G., Philippe, S., Zayed, J., 2003. Bioaccumulation and locomotor effects of manganese phosphate/sulfate mixture in Sprague–Dawley rats following subchronic (90 days) inhalation exposure. Toxicol. Appl. Pharmacol. 191, 264–271.

Semenza, G.L., 2005. Involvement of hypoxia-inducible factor 1 in pulmonary pathophysiology. Chest 128, 592S–594S.

Sugihara, T., Wadhwa, R., Kaul, S.C., Mitsui, Y., 1998. A novel alternatively spliced form of murine vasculare endothelial growth factor, VEGF 115. J. Biol. Chem. 273, 3033–3038.

Vengellur, A., LaPres, J.J., 2004. The role of hypoxia inducible factor 1α in cobalt chloride induced cell death in mouse embryonic fibroblasts. Toxicol. Sci. 82, 638–646.

Vitarella, D., Moss, O., Dorman, D.C., 2000. Pulmonary clearance of manganese phosphate, manganese sulfate, and manganese tetroxide by CD rats following intratracheal instillation. Inhal. Toxicol. 12, 941–957.

Voelkel, N.E., Vandivier, R.W., Tuder, R.M., 2006. Vascular endothelial growth factor in the lung. Am. J. Physiol. Lung Cell. Mol. Physiol. 290, L209–L221.

Wang, G.L., Jiang, B.H., Rue, E.A., Semenza, G.L., 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514.

Yuan, Y., Hilliard, G., Ferguson, T., Millhorn, D.E., 2003. Cobalt inhibits the interaction between hypoxia-inducible factor-α and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-α. J. Biol. Chem. 278, 15911–15916.