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Manganese Potentiates Nuclear Factor- κ B-Dependent Expression of Nitric Oxide Synthase 2 in Astrocytes by Activating Soluble Guanylate Cyclase and Extracellular Responsive Kinase Signaling Pathways

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Abstract

Inflammatory activation of glial cells is associated with neuronal injury in several degenerative movement disorders of the basal ganglia, including manganese neurotoxicity. Manganese (Mn) potentiates the effects of inflammatory cytokines on nuclear factor- κ B (NF- κ B)-dependent expression of nitric oxide synthase 2 (NOS2) in astrocytes, but the signaling mechanisms underlying this effect have remained elusive. It was postulated in the present studies that direct stimulation of cGMP synthesis and activation of mitogen-activated protein (MAP) kinase signaling pathways underlies the capacity of Mn to augment NF- κ B-dependent gene expression in astrocytes. Exposure of primary cortical astrocytes to a low concentration of Mn (10 μ M) potentiated expression of NOS2 mRNA and protein along with production of NO in response to interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α), which was prevented by overexpression of dominant negative I κ B α . Mn also potentiated IFN γ - and TNF α -induced phosphorylation of extracellular response kinase (ERK), p38, and JNK, as well as cytokine-induced activation of a fluorescent NF- κ B reporter construct in transgenic astrocytes. Activation of ERK preceded that of NF- κ B and was required for maximal activation of NO synthesis. Independently of IFN γ /TNF α , Mn-stimulated synthesis of cGMP in astrocytes and inhibition of soluble guanylate cyclase (sGC) abolished the potentiating effect of Mn on MAP kinase phosphorylation, NF- κ B activation, and production of NO. These data indicate that near-physiological concentrations of Mn potentiate cytokine-induced expression of NOS2 and production of NO in astrocytes via activation of sGC, which promotes ERK-dependent enhancement of NF- κ B signaling.

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Keywords

astrocyte; manganese; inducible nitric oxide synthase; nuclear factor- κ B; extracellular response kinase; soluble guanylate cyclase

Manganese (Mn) is an essential cofactor for multiple enzymes critical to metabolic homeostasis in the central nervous system (CNS), including mitochondrial superoxide dismutase (Hearn et al., 2003) and glutamine synthetase (Takeda, 2003). However, excessive accumulation of Mn can cause injury to neurons in the basal ganglia, resulting in a progressive neurodegenerative disorder known as *manganism*, which is accompanied by reactive astrogliosis (Yamada et al., 1986). Studies using rodent models of manganism suggest that astrocytes are affected early in Mn neurotoxicity, based on changes in expression of the phenotypic markers of astrocyte activation, glial fibrillary acidic protein (GFAP) and S100 β , that precede overt neuronal injury (Henriksson and Tjälve, 2000). Additionally, astrocytes accumulate much higher levels of Mn than neurons (Maurizi et al., 1987), which may disrupt critical neurotrophic functions and promote an inflammatory phenotype. Mn enhances the release of inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α) from microglial cells (Chang and Liu, 1999; Filipov et al., 2005), which can promote the activation of astrocytes and subsequent release of inflammatory mediators such as prostaglandin E₂ and nitric oxide (NO; Hirsch et al., 1998; Spranger et al., 1998; Chen et al., 2006). Previous studies from our laboratory (Liu et al., 2005) and others (Spranger et al., 1998) demonstrated that Mn strongly potentiates NO production in cytokine-stimulated astrocytes, leading to apoptosis in cocultured neurons. Low concentrations of Mn can potentiate the capacity of TNF α and IL-1 β to induce expression of NOS2 and production of NO in astrocytes by acting on nuclear factor- κ B (NF- κ B; Spranger et al., 1998; Liu et al., 2005), but the signaling mechanisms responsible have not been elucidated. NO is produced from L-arginine by nitric oxide synthases (NOS1, -2, and -3), the inducible isoform of which (NOS2) is not constitutively expressed but is highly up-regulated in activated astrocytes and microglia by stimuli such as proinflammatory cytokines and bacterial lipopolysaccharide (LPS; Stuehr and Griffith, 1992).

Expression of NOS2 is regulated principally by the transcription factor NF- κ B (Xie et al., 1993), a Rel protein family member involved in inflammation, cell division, apoptosis, and immune responses (Karin and Ben-Neriah, 2000; Perkins, 2000). Signals that activate NF- κ B converge through the I κ B kinase (IKK) complex to phosphorylate I κ B α , targeting it for degradation by 26S proteasome and allowing the p65-RelA/p50 subunits of NF- κ B to translocate into the nucleus (Nakano et al., 1998; Vermeulen et al., 2002). Upstream activators of NF- κ B include mitogen-activated protein (MAP) kinases, such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-responsive kinase (ERK; Kim et al., 2006; Fernandes et al., 2007). We previously demonstrated that Mn-dependent activation of ERK in C6 glioma cells augments LPS-induced expression of NOS2 through the NF- κ B pathway (Barhoumi et al., 2004), but a similar mechanism has not been reported for astrocytes. One possible target of Mn in astrocytes is the NO-sensitive soluble guanylate cyclase (sGC) that catalyzes the conversion of GTP to cyclic GMP (cGMP; Sardon et al., 2004; Shen et al., 2005). It has been reported with other systems that the enzymatic activity of sGC is strongly

increased in the presence of Mn^{2+} to a greater extent than its native cofactor, Mg^{2+} (Winger and Marletta, 2005). Interestingly, expression of sGC is increased in the striatum of mice exposed to the parkinsonian neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Chalimoniuk et al., 2004). These data are intriguing given the established role for glial inflammation in the neurotoxicity of MPTP, but a similar role for cGMP in Mn neurotoxicity remains to be determined. We postulated, based on these findings and those of previous studies from our laboratory implicating NF- κ B in Mn-dependent expression of NOS2 (Barhoumi et al., 2004; Liu et al., 2005), that Mn potentiates cytokine-induced activation of NF- κ B and expression of NOS2 in astrocytes by directly enhancing production of cGMP that stimulates MAP kinase signaling.

MATERIALS AND METHODS

Reagents

All chemical reagents were obtained from Sigma (St. Louis, MO), unless otherwise indicated. C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Fluorescent dyes, cell culture media, antibiotics, and Alexa-Fluor secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

Primary cortical murine astrocytes were isolated from cortices of 1-day-old C57/Bl6 and transgenic GFP mice as described by Aschner et al. (1992) and grown 18 days to maturity before use in experiments. Astrocytes were maintained in minimum essential medium (MEM) with L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin (PSN) at 37°C, 5% CO₂ in a humidified atmosphere. Immunostaining for GFAP indicated that cultures were consistently >96% astrocytes.

NOS2 mRNA and Protein Expression

RNA was isolated by using the Qiagen RNeasy Kit, with on-column DNase digestion, and reverse transcribed via iScript (Bio-Rad, Hercules, CA). Semiquantitative real-time PCR was used to measure relative *Nos2* expression normalized to β -*actin* mRNA. The following primers designed from mouse *Nos2* and β -*actin* transcript were used: *Nos2* (NCBI accession No. P29477) 5'-TCACGCTTGGGTCTTGTT-3' (forward), 5'-CAGGTCACCTTTGGTAGGATTTG-3' (reverse); β -*actin* 5'-GCTGTGCTATGTTGCTCTAG-3' (forward), 5'-CGCTCGTTGCCAATAGTG-3' (reverse). NOS2 protein expression levels in astrocytes were measured by immunofluorescence. Briefly, astrocytes plated on glass coverslips underwent methanol fixation, were permeabilized with 0.01% Triton X-100, and then were probed for the protein of interest. Primary monoclonal antibody for GFAP and polyclonal antibodies for NOS2 (BD Pharmingen, San Deigo, CA) and phosphorylated ERK (Cell Signaling, Danvers, MA) were used at 1:1,000, 1:400, and 1:100, respectively. Protein levels were visualized via fluorescence microscopy following hybridization of Alexa Fluor 488-or 647-labeled secondary antibodies.

Determination of NO Steady-State Levels

NO production was assessed by wide-field live-cell fluorescence imaging in primary astrocytes exposed to treatment groups for 8 hr and then incubated with the fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate prepared as a 5 mM stock in dimethylsulfoxide (DMSO) and diluted to a final concentration of 5 μ M in medium for 10 min. Images were then collected at 490 nm excitation/520 nm emission at intervals of 5 min for 60 min using a Zeiss Axiovert 200M microscope equipped with a Hamamatsu ORCA-ER cooled charge-coupled device camera.

Adenoviral Expression in Astrocytes

To determine the upstream role of NF- κ B with Mn and cytokines exposure, activation of NF- κ B was suppressed by a mutant NF- κ B inhibitory subunit (I κ B α) containing serine-to-alanine mutations at amino acids 32 and 36. This construct has been subcloned into an adenoviral vector (provided by Dr. David Brenner, MD, Professor of Medicine, Columbia University) and is expressed in 90–100% of infected astrocytes at 1×10^3 viral particles per milliliter of culture media for 24 hr. After incubation of virus for 24 hr, medium was removed, cells were washed with PBS to remove viral particles, and culture medium was replaced for 24 hr prior to treatment. Parallel control experiments were performed using the adenoviral vector lacking the mtI κ B α insert as described previously (Liu et al., 2005).

Immunoblotting of Phosphorylated MAP Kinases

Mature astrocytes were serum starved for two hr followed by treatment with Mn and cytokines. Protein was then isolated by lysing cells using RIPA buffer containing sodium orthovanadate (0.2 mM) and Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein, 10 or 20 μ g, was resolved by 10% SDS-PAGE. Resolved proteins were transferred to polyvinylidene fluoride membranes and incubated with primary polyclonal antibodies, phospho-ERK, phospho-JNK, or phospho-p38 at 1:4,000, 1:1,000, and 1:1,000, respectively (Cell Signaling). Horseradish peroxidase-conjugated secondary antibody (1:5,000; Cell Signaling) was applied, and protein was visualized by using chemiluminescence (Amersham Biosciences, Piscataway, NJ). The membrane was then reprobed with a protein loading control, total ERK 1:5,000, total JNK 1:1,000, and total p38 1:1,000 (Cell Signaling).

cGMP and ERK Activation Assays

To assay guanosine 3',5'-cyclic monophosphate (cGMP) levels with a competitive enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI), astrocytes were treated and then lysed with 0.1 M hydrogen chloride. Samples were acetylated to achieve quantification of cGMP at concentrations less than 1 pmol/ml. Each treatment group had three biological replicates, and each replicate was assayed in triplicate. The assay was analyzed using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) measuring absorbance at 420 nm. A Cellular Activation of Signaling ELISA (CASE) kit, a colorimetric assay, was utilized to measure total ERK and ERK phosphorylation (Superarray, Frederick MD). Cells were plated on a 96-well tissue culture plate at 5×10^4 cells per well, then treated in duplicate representing three biological replicates. Quantitative analysis of both

phosphorylated and total ERK was performed using the aforementioned microplate reader at absorbance 450 nm.

Statistical Analysis

At least three repetitions per isolation of each sample group were performed for each study, consisting of three separate biological replicates. Two-group comparison was performed utilizing Student's *t*-test, and one-way ANOVA with Neuman-Keuls post hoc test was used for comparing differences among more than three means. Differences between groups were considered significant at $P < 0.05$ and indicated through assignment of a unique character (e.g., a–d).

RESULTS

Expression of *Nos2* mRNA was examined in primary mouse cortical astrocytes exposed to low concentrations of Mn and IFN γ /TNF α to identify a threshold dose that potentiated cytokine-mediated induction of this gene. Treatment with 10 μ M Mn led to a slight increase in levels of *Nos2* mRNA (2.5 ± 0.8 -fold greater than control; Fig. 1A). Combinatorial treatment with IFN γ and TNF α resulted in a dose-dependent increase in *Nos2* mRNA levels (Fig. 1B), with 1,000 pg/ml IFN γ + 10 pg/ml TNF α causing a 8.0 ± 3.0 -fold increase over control, which was selected as a “suboptimal” dose corresponding to mild activation of astrocytes. Exposure of astrocytes to 10 μ M Mn in the presence of 1,000 pg/ml IFN γ + 10 pg/ml TNF α strongly potentiated induction of *Nos2* mRNA levels (21.3 ± 9 -fold increase over control; Fig. 1C). Based on these data, this dose of Mn and cytokines was used in subsequent experiments to induce an inflammatory phenotype and expression of *Nos2*. Time-dependent expression of *Nos2* mRNA levels in astrocytes exposed to 10 μ M Mn + IFN γ /TNF α for 0, 2, 4, 6, and 8 hr indicated that expression of message significantly increased by 8 hr (Fig. 1D).

Expression of NOS2 protein was determined in astrocytes by immunofluorescence following 8 hr of exposure to 10 μ M Mn + IFN γ /TNF α (Fig. 2). As shown in Figure 2A, expression of NOS2 (red fluorescence) was not detected in control cells but was induced upon exposure to Mn + IFN γ /TNF α . In all fields examined, expression of NOS2 was localized to cells expressing GFAP (green fluorescence). Production of NO was assessed by live cell imaging using the fluorescent NO indicator DAF-FM diacetate in primary astrocytes exposed to Mn + IFN γ /TNF α for 8 hr. Intracellular levels of NO were increased by $6\% \pm 0.2\%$ and $4\% \pm 0.06\%$ in astrocytes exposed to 10 μ M Mn or 1,000 pg/ml IFN γ + 10 pg/ml TNF α , respectively, but increased to $18.7\% \pm 5.7\%$ over control in cells exposed to Mn + IFN γ /TNF α (Fig. 2C).

The role of NF- κ B in Mn-induced expression of NOS2 is examined in Figure 3. Primary astrocytes were transfected with a control adenoviral vector or with adenovirus expressing a phosphorylation-deficient mutant of I κ B α (mtI κ B α ; I κ B α S32/36A) and exposed to Mn + IFN γ /TNF α . Expression of mtI κ B α partially abrogated induction of *Nos2* mRNA upon treatment with Mn + IFN γ /TNF α , whereas *Nos2* mRNA levels were increased similarly to untransfected astrocytes in cells exposed to Mn + IFN γ /TNF α in the presence of control adenoviral vector (Fig. 3A). Exposure to Mn + IFN γ /TNF α increased NOS2 protein in

control-transfected astrocytes, and this induction was completely prevented in cells expressing mtI κ B α (Fig. 3B). Similarly, overexpression of mtI κ B α prevented Mn- and IFN γ /TNF α -induced increases in steady-state intracellular levels of NO, as determined by live-cell imaging (Fig. 3C). Treatment with Mn + IFN γ /TNF α also directly increased activation of NF- κ B in transgenic astrocytes expressing an NF- κ B-GFP reporter construct; green fluorescence in these cells represents functional transactivation by p65 (Fig. 3D, inset and graph). As a control, transgenic astrocytes were transfected with mtI κ B α or control vector and assessed for increased GFP fluorescence (Fig. 3E). Treatment with Mn + IFN γ /TNF α strongly enhanced GFP fluorescence in transgenic astrocytes transfected with control vector, whereas expression of mtI κ B α prevented both basal and inducible induction of GFP.

Mn induced rapid phosphorylation of the MAP kinase family members ERK, JNK, and p38 (Fig. 4). Phosphorylation of ERK increased only slightly over time in astrocytes treated with either 10 μ M Mn or IFN γ + TNF α individually, but coexposure potentiated phosphorylation of ERK, which was maximal at 120 min (Fig. 4A). Treatment with Mn + IFN γ /TNF α also increased time-dependent phosphorylation of p38, but JNK phosphorylation occurred in a cyclic manner, with increased phosphorylation at 5 and 10 min, followed by a decrease at 30 min (Fig. 4B,C). To determine the functional significance of Mn-dependent MAP kinase activation, production of NO was determined by live-cell fluorescence imaging in astrocytes exposed to Mn + IFN γ /TNF α in the presence of inhibitors of ERK, p38, and JNK (Fig. 4D). Inhibition of ERK (U0126, 10 μ M) and p38 (SB203580, 30 μ M) attenuated NO production following exposure to Mn + IFN γ /TNF α , but inhibition of JNK (SP600125, 10 μ M) failed to decrease NO production induced by Mn + IFN γ /TNF α . NO levels were significantly increased in the presence of vehicle control (DMSO) comparably to treatment with Mn + IFN γ /TNF α with inhibitors for ERK and p38, indicating that the inhibition of NO production was due solely to the actual inhibitor and not to the vehicle, DMSO.

The temporal sequence of ERK and NF- κ B activation was determined in transgenic NF- κ B reporter astrocytes by coimmunofluorescence following exposure to Mn and IFN γ /TNF α (Fig. 5). The fluorescence intensity of phosphorylated ERK in transgenic NF- κ B-GFP astrocytes peaked at 4 hr, followed by maximal NF- κ B activation at 6 hr (Fig. 5A). Quantitation of fluorescence imaging data (Fig. 5B) indicated significant differences from control for both ERK and NF- κ B upon exposure to Mn and IFN γ /TNF α . By 2 hr, levels of phosphorylated ERK increased, peaking at 4 hr and subsiding to baseline by 6 hr. NF- κ B activity, as determined by GFP fluorescence, was not increased until 6 hr and decreased to a level still greater than control by 8 hr. The functional correlation between Mn-dependent activation of ERK and NF- κ B was determined by immunoblotting for phosphorylated I κ B α in astrocytes exposed to concentrations of Mn from 0 to 50 μ M in the absence or presence of the ERK inhibitor U0126 (Fig. 5C). Mn directly enhanced the phosphorylation of I κ B α at all concentrations examined, and this effect was prevented by U0126 (10 μ M).

Synthesis of cGMP by sGC was next examined as a potential target of Mn leading to activation of ERK and NF- κ B (Fig. 6). Levels of cGMP were determined by enzyme-linked immunosorbent assay (ELISA) in astrocytes treated with 0, 1, 10, and 100 μ M MnCl $_2$ in the absence or presence of IFN γ /TNF α (Fig. 6A). Mn caused a dose-dependent increase in levels of cGMP at concentrations as low as 1 μ M (218 ± 53 pmol/ml vs. 124 ± 11 pmol/ml,

control) that were near maximal at 10 μ M (380 ± 44 pmol/ml), with 100 μ M Mn increasing cGMP levels to only 456 ± 15 pmol/ml. Treatment with IFN γ /TNF α in the absence of Mn increased cGMP over control levels but did not enhance Mn-induced increases in cGMP (Fig. 6A, final two columns). The EC₅₀ for Mn-dependent activation of cGMP production in astrocytes was 1.1 ± 0.4 μ M (Fig. 6A, inset). The sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; EMD Biosciences) prevented increases in cGMP induced by 10 μ M Mn + IFN γ /TNF α (412 ± 43 pmol/ml, Mn + IFN γ /TNF α vs. 122 ± 8 pmol/ml, Mn + IFN γ /TNF α + ODQ), but vehicle control (DMSO) had no effect on Mn-dependent activation of sGC (Fig. 6B). Inhibition of sGC with ODQ also prevented Mn-dependent activation of ERK, as determined by using ELISA to measure the amount of phosphorylated and total ERK in cellular lysates (Fig. 6C). Levels of phosphorylated ERK were suppressed by ODQ in astrocytes exposed to Mn and IFN γ /TNF α (Fig. 6B), whereas levels of total ERK remained unchanged (Fig. 6D). Finally, cotreatment with ODQ but not DMSO significantly inhibited NO production (Fig. 6E) and NF- κ B activation (Fig. 6F) in live astrocytes following exposure to Mn and IFN γ /TNF α .

DISCUSSION

Increased production of NO by reactive glia has been linked to the progression of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases and manganism (Hunot et al., 1999; Gomez-Isla et al., 2003; Hirsch et al., 2003). Accordingly, gene deletion of *Nos2* in mice is neuroprotective in models of ischemia (Sugimoto and Iadecola, 2002) and parkinsonian neurodegeneration (Liberatore et al., 1999), but a direct role for NO in manganism is less clear. We reported recently that loss of striatal interneurons in Mn-exposed mice was most prominent near reactive astrocytes expressing NOS2 and was associated with increased immunoreactivity for 3-nitrotyrosine protein adducts, a marker of peroxynitrite (ONOO⁻) formation (Liu et al., 2006). Additionally, we reported that expression of NOS2 and overproduction of NO in activated astrocytes exposed to Mn causes apoptosis in cocultured PC12 cells (Liu et al., 2005).

NF- κ B is directly associated with overproduction of NO in Mn-treated astrocytes (Barhoumi et al., 2004; Liu et al., 2005), but the mechanism underlying the capacity of Mn to potentiate the effects of inflammatory cytokines and LPS- on NF- κ B-dependent induction of NOS2 has remained elusive. The MAP kinase family of stress-activated protein kinases can directly activate NF- κ B through the IKK signaling complex (Lee et al., 1998; Nakano et al., 1998) and represent a potential upstream signaling target underlying Mn-dependent activation of NF- κ B. The data presented here indicate that Mn enhances cytokine-induced activation of NF- κ B and expression of NOS2 through the ERK pathway. Additionally, these data reveal that the mechanism by which Mn stimulates ERK requires increases in cGMP. Convergence of Mn- and cytokine-induced signaling pathways resulted in robust activation of MAP kinases (Fig. 4). The cyclic nature of phosphorylation of both ERK and JNK shown in Figure 4A,C is commonly reported both basally and during stress-induced signaling (Bae et al., 2006; Chen et al., 2007). Physiological concentrations of Mn range from 2 to 8 μ M in brain tissue (Pal et al., 1999), and, at 10 μ M, Mn significantly elevated intracellular levels of cGMP in astrocytes, which resulted in rapid activation of ERK (Fig. 6). Interestingly, we found in previous studies that individual treatment with low levels of Mn or IFN γ /TNF α did

not significantly induce expression of NOS2 (Liu et al., 2005), and the results here suggest that the capacity of Mn to potentiate inflammatory expression of NOS2 resides in its potent effect on activation of cGMP production.

These observations provide a mechanistic explanation for earlier findings describing the ability of Mn to enhance NF- κ B-dependent expression of NOS2 in activated astrocytes (Spranger et al., 1998; Liu et al., 2005). Genetic inhibition of NF- κ B through overexpression of mtI κ B α completely prevented Mn-dependent increases in NOS2 protein and NO (Fig. 3) while only partially preventing increases in *Nos2* mRNA. This partial abrogation of *Nos2* mRNA levels may be attributed to the possible contribution of other transcription factors that can induce expression of *Nos2*, such as signal transducer and activator of transcription-1 alpha (STAT-1 α), cAMP-responsive element binding protein (CREB), and activating protein-1 (AP-1; Lee et al., 2003; De Stefano et al., 2006; Blanchette et al., 2007). A prior study by Chen et al. (2006) demonstrated that Mn enhanced AP-1 activity in mixed glia cultures primed with LPS and IFN γ , indicating that this transcription factor could also be involved in Mn-induced expression of *Nos2*. However, the observation that steady-state levels of NOS2 protein and NO were both decreased to control levels by mtI κ B α , despite the incomplete suppression of transcript levels, suggests that NF- κ B might also influence posttranscriptional regulation of NOS2 expression. This is supported by data demonstrating that NOS2 requires the cofactor tetrahydrobiopterin for full protein assembly and activity (Chiarini et al., 2005) and by studies reporting that the rate-limiting enzyme in the synthesis of tetrahydrobiopterin, GTP cyclohydrolase, is coordinately regulated with NOS2 by an NF- κ B-dependent mechanism (Togari et al., 1998; Cho et al., 2001). Additional preliminary studies from our laboratory indicate that Mn potentiates cytokine-induced expression of GTP cyclohydrolase mRNA and that this induction is suppressed by mtI κ B α (data not shown).

The present studies indicate that concentrations of Mn only slightly above physiologic levels can strongly potentiate inflammatory signaling pathways in astrocytes primed with low levels of cytokines. This may provide insight into why astrogliosis is so prominently associated with neurodegeneration in humans (Yamada et al., 1986) and experimental animals (Spranger et al., 1998; Liu et al., 2006) exposed to Mn. Brain Mn levels increase overall only two- to threefold within the basal ganglia in models of manganism (Liu et al., 2006), but astrocytes can accumulate concentrations 50-fold greater than neurons (Maurizi et al., 1987; Aschner et al., 1992) as a result of active transport systems (Aschner et al., 1992), easily within the range shown here that stimulates sGC activity and MAP kinase signaling. Through the use of transgenic astrocytes expressing an NF- κ B reporter construct, the data in Figure 3 demonstrate not only that NF- κ B is required for expression of NOS2 but also that low concentrations of Mn enhance functional transactivation of NF- κ B through p65 consensus binding elements. Previous data from our laboratory suggested that ERK was a potential target of Mn and that convergent signaling from this pathway enhanced NF- κ B activation (Barhoumi et al., 2004). The data presented here demonstrate both that activation of ERK precedes the peak of NF- κ B activity (Fig. 5) and that cGMP-dependent activation of ERK is required for Mn to potentiate expression of NOS2 and production of NO in cytokine-primed astrocytes (Fig. 6). Experiments examining activity of MAP kinases and

production of NO in the presence of various kinase inhibitors (Fig. 4) indicated that p38 has some capacity to stimulate NO production independently of ERK. However, the efficacy of $\text{mtI}\kappa\text{B}\alpha$ in completely preventing Mn-dependent increases in NOS2 protein and NO levels suggests that activation of p38 in this system also likely conveys Mn-dependent signals directly to the IKK/NF- κ B complex (Fig. 4D). This is consistent with other studies reporting direct activation of NF- κ B by p38 (Kim et al., 2006) and with reports indicating that inhibitors of p38 prevent Mn-dependent expression of TNF α in microglial cells (Filipov et al., 2005), a gene also strongly induced by NF- κ B. However, p38-dependent activation of other transcription factors, such as JAK/STAT proteins, could partially explain the slightly elevated levels of *Nos2* mRNA that persist even in the presence of overexpressed $\text{mtI}\kappa\text{B}\gamma$, despite complete suppression of NOS2 protein.

The data in Figure 6 identifying sGC as a direct target of Mn in astrocytes provide a critical link explaining how only slightly elevated concentrations of Mn augment stress kinase signaling that subsequently potentiates activation of NF- κ B. The mechanism by which Mn activates sGC is reported to involve replacement of the native cofactor Mg^{2+} , which increases activity of the enzyme (Braugher, 1980; Winger and Marletta, 2005). The relevance of increased sGC to glial activation and neurodegeneration in manganism has not been reported. However, an association is suggested by indirect evidence from studies in MPTP-treated mice reporting an increase in expression of sGC and levels of cGMP activity in the striatum (Chalimoniuk et al., 2004). Concentrations of Mn as low as 10 μM increased cGMP levels in cultured astrocytes (Fig. 6A). The addition of low concentrations of IFN γ and TNF α did not further enhance cGMP levels upon combined exposure with Mn (Fig. 6B), indicating that the observed increase in cGMP was Mn dependent and distinct from signaling pathways activated by inflammatory cytokines. Additionally, inhibition of sGC prevented activation of both ERK and NF- κ B, as well as production of NO (Fig. 6C–F), in Mn-treated astrocytes, indicating a sequence of signaling events involving sGC/cGMP/ERK that converges on the IKK/NF- κ B pathway to potentiate expression of NOS2 in astrocytes.

In conclusion, the present studies demonstrate that near-physiological levels of Mn strongly induce NOS2 and production of NO in astrocytes primed by a mild inflammatory insult. The mechanism underlying the striking effect of low-level Mn on expression of NOS2 in astrocytes appears to reside in the capacity of the divalent metal to stimulate sGC potently, leading to elevated intracellular levels of cGMP and ERK-dependent activation of NF- κ B. Further dissection of the signaling pathways leading to activation of MAP kinases in Mn-exposed astrocytes, as well as the downstream transcriptional mechanisms regulating *trans*-activation by NF- κ B, will likely improve our understanding of how NOS2 and other inflammatory genes are inappropriately expressed in activated astroglia during degenerative conditions of the basal ganglia such as manganism.

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Fig. 1.

Manganese potentiates cytokine-induced expression of *Nos2* mRNA in astrocytes. **A:** *Nos2* mRNA expression was measured by using real-time PCR in astrocytes exposed to saline or 0.1 μM , 1 μM , or 10 μM MnCl_2 (Mn) for 8 hr. *Nos2* mRNA expression was significantly increased when astrocytes were treated with 10 μM Mn compared with control. **B:** *Nos2* mRNA expression in cells exposed to saline or 100 pg/ml IFN γ + 1 pg/ml TNF α , 1,000 pg/ml IFN γ + 10 pg/ml TNF α , or 10,000 pg/ml IFN γ + 100 pg/ml TNF α for 8 hr indicated that 1,000 pg/ml IFN γ + 10 pg/ml TNF α concentration is adequate to induce moderate

expression. **C:** Astrocytes were exposed to 1,000 pg/ml IFN γ + 10 pg/ml TNF α (I/T) and 0–10 μ M of Mn for 8 hr, showing potentiation of *Nos2* mRNA expression with exposure to 10 μ M Mn + I/T. **D:** Astrocytes exposed to saline or 10 μ M Mn + I/T over time (0–8 hr), with increased *Nos2* mRNA expression observed at 4, 6, and 8 hr.

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Fig. 2.

Manganese potentiates cytokine-induced expression of NOS2 protein and production of NO in astrocytes. **A:** Astrocytes were exposed to saline or 10 μ M Mn, 1,000 pg/ml IFN γ + 10 pg/ml TNF α (I/T), or Mn + I/T for 8 hr and analyzed by immunofluorescence for expression of NOS2 protein. Representative images showing GFAP (green) and NOS2 (red) protein levels. **B:** Quantification of NOS2 fluorescence indicating significantly increased protein expression upon exposure to Mn + I/T compared with control in GFAP positive cells. **C:** Astrocytes were exposed to Mn + I/T as in A, and NO levels were determined by live-cell

fluorescence imaging using the NO indicator DAF-FM. NO production was significantly increased by Mn and I/T individually and strongly potentiated by combinatorial treatment with Mn + I/T. Scale bar = 10 μ m.

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Fig. 3.

Manganese-induced potentiation of NOS2 expression and production of NO requires activation of NF- κ B. Astrocytes were exposed for 8 hr to saline or 10 μ M Mn, 1,000 pg/ml IFN γ + 10 pg/ml TNF α (I/T), or Mn + I/T in the absence or presence of a dominant negative mutant of I κ B α (mtI κ B α -S32/36A) and examined for NOS2 expression and NO production. **A:** Mutant I κ B α significantly decreased expression of *Nos2* mRNA in astrocytes exposed to Mn + I/T compared with control vector. **B:** Expression of NOS2 protein was prevented by mutant I κ B α following exposure to Mn + I/T compared with the control vector treatment

group. **C:** Astrocytes were exposed to Mn + I/T, and NO levels were determined by live-cell fluorescence imaging using the NO indicator DAF-FM. Expression of mutant I κ B γ prevented increases in NO in astrocytes exposed to Mn + I/T compared with control vector. **D:** Inset: Schematic representation of NF- κ B-enhanced green fluorescent protein (EGFP) reporter construct in transgenic astrocytes. Astrocytes were exposed to Mn + I/T, and the total fluorescence intensity per cell was determined by live-cell imaging. NF- κ B activation is significantly increased by Mn + I/T. **E:** NF- κ B activation is decreased upon exposure of Mn + I/T in the presence of mutant I κ B α compared with empty vector. Data represent at least three independent experiments. Different letters denote significant differences between groups. In A, ab in column 3 denotes no significant difference from columns 1 and 2. In B, ac in column 4 denotes no significant difference from columns 1 and 3.

Fig. 4. MAP kinase activation in Mn-treated astrocytes is required for potentiation of NO production. Phosphorylation of ERK, p38, and JNK was measured by immunoblotting in primary astrocytes treated with saline control or 10 μM Mn, 1,000 pg/ml IFNγ + 10 pg/ml TNFα (I/T), or Mn + I/T in serum-free media **A:** Phosphorylation of ERK (P-ERK) was assessed in astrocytes exposed to saline, Mn, I/T, or Mn + I/T over time (0–120 min). **B:** Phospho-p38 (P-p38) in astrocytes treated with saline and Mn + I/T. **C:** Phospho-JNK levels (P-JNK) in astrocytes exposure to saline or Mn + I/T. Mn enhanced time-dependent

phosphorylation of ERK, JNK, and p38. Membranes were reprobed for total ERK, p38, and JNK to control for protein loading. **D:** NO levels in astrocytes exposed to Mn + I/T were determined in the presence of inhibitors for ERK (U0126; 10 μ M), p38 (SB203580; 30 μ M), and JNK (SP600125; 10 μ M). Inhibition of ERK and p38, significantly inhibited NO production.

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Fig. 5.

ERK is required for Mn-dependent activation of NF- κ B. **A:** Activation of ERK and NF- κ B was simultaneously determined over time in NF- κ B-GFP transgenic astrocytes by immunofluorescence. Images of transgenic NF- κ B-GFP astrocytes exposed to 10 μ M Mn and 1,000 pg/ml IFN γ + 10 pg/ml TNF α (Mn + I/T) at 0, 2, 4, 6, or 8 hr depicting activation of NF- κ B (green), phosphorylation of ERK (P-ERK, red), nuclear staining (DAPI, blue), and merged images. **B:** Quantitative analysis of P-ERK and NF- κ B-GFP over time indicates that phosphorylation of ERK peaks at 4 hr, preceding the peak of NF- κ B activation at 6 hr. Groups were compared within each fluorescence channel by one-way ANOVA, followed by Neuman-Keuls test ($P < 0.05$). **C:** Immunoblot for phosphorylation of I κ B α (P-I κ B α) in astrocytes exposed to 0–50 μ M Mn, U0126 (10 μ M), or 50 μ M Mn + U0126 (10 μ M). Blots

were reprobed for total I κ B γ to control for protein loading and are representative of three independent experiments. Scale bar = 10 μ m.

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Fig. 6. Soluble guanylate cyclase (sGC) relays signals to ERK and NF- κB in Mn-treated astrocytes. **A:** Astrocytes were treated for 8 hr with 0, 1, 10, and 100 μM Mn or 10 and 100 μM Mn + 1,000 pg/ml IFN γ and 10 pg/ml TNF α (I/T), and cGMP levels were determined by ELISA. Mn significantly increased intracellular cGMP at 10 and 100 μM , which was not further enhanced by cotreatment with I/T. The EC_{50} of Mn-induced cGMP production in astrocytes was 1.1 ± 0.4 pmol/ml (**inset**). **B:** Mn-induced increases in cGMP were inhibited by the sGC inhibitor ODQ (10 μM) following treatment with 10 μM Mn + I/T but not by vehicle control

(DMSO). **C:** Phosphorylation of ERK was determined by ELISA and was significantly decreased in astrocytes exposed to 10 μ M Mn + I/T + ODQ compared with 10 μ M Mn + I/T alone or 10 μ M Mn + I/T + DMSO. **D:** Total levels of ERK did not change among the treatment groups in C. **E:** ODQ significantly decreased NO levels in astrocytes exposed to 10 μ M Mn + I/T compared with 10 μ M Mn + I/T alone or 10 μ M Mn + I/T + DMSO. **F:** Transgenic NF- κ B-GFP astrocytes were exposed to 10 μ M Mn + I/T with and without DMSO or ODQ; ODQ prevented activation of NF- κ B upon treatment with Mn + I/T. Different letters denote significant differences between groups. In A, ac in column 2 denotes no significant difference from columns 1 and 5, bc in column 5 denotes no significant difference from columns 2–4 and 6.