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Potentiation of microglial toll-like receptor stimulated inflammatory cytokine output by manganese: a role for p38 mitogen-activated protein kinase

Patrick L. Crittenden

POTENTIATION OF MICROGLIAL TOLL-LIKE RECEPTOR STIMULATED INFLAMMATORY CYTOKINE OUTPUT BY MANGANESE: A ROLE FOR P38 MITOGEN-ACTIVATED PROTEIN KINASE

By

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Manganese (Mn) neurotoxicity has been recognized for a long time and it affects primarily the basal ganglia. However, only recently has inflammation, which is known to play an important role in neuropathology, been considered as part of its toxic mechanism. In the current study, we have shown that Mn-potentiation of inflammatory cytokines (IL-6 and TNF-α) in microglia is not dependent on the activation of the Toll-like receptor (TLR)-4 signaling pathway by lipopolysaccharide (LPS), as potentiation by Mn is observed in microglia activated with agonists for other TLR, such as TLR2, TLR3, and TLR9. Furthermore, enhancement of inflammatory cytokine production by Mn is independent of the MyD88 signaling pathway, as Mn-potentiation

in TLR3-activated microglia occurs by a signaling pathway which is MyD88-independent.

Additionally, inhibition of p38, ERK, or JNK mitogen-activated protein kinases (MAPK) prevented Mn-potentiation, which suggest that these MAPK are required for potentiation of inflammatory cytokines by Mn. We also observed that Mn exposure results in persistent activation of p38. The prolonged activation of p38 may be due to increased activation of the 'upstream' MAPK, mitogen-activated protein kinase kinase (MKK)-1/2 or MKK-4, or to decreased expression of the phosphatase, mitogen-activated protein kinase phosphatase-1 (MKP-1), that is responsible for deactivating p38.

Overall, it appears that Mn potentiates microglial inflammatory cytokine output in conjunction with diverse stimuli by an unknown mechanism, but likely involving MAPK and perhaps extending their activation, that of p38 in particular. This ability of Mn to potentiate brain inflammation should be considered as part of its neurotoxic mechanism.

DEDICATION

I would to dedicate this research to my parents, Ray and Mary Crittenden, for their support and encouragement while I have pursued my graduate education.

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CHAPTER I

INTRODUCTION

Overview of metal toxicity

The use of metals has been an important facet of human society since the end of the Stone Age of man. From tools for agriculture to weapons of war, metals have been applied to many uses, including therapeutics for ailments and disease. Indeed, it is perhaps the medicinal uses that first illustrated the toxicity that could occur from prolonged or excessive metal exposures (Koehler, 2001). Metal organ system toxicities include adverse effects of cadmium on the endocrine and renal systems (Fowler, 1993; Henson and Chedrese, 2004), and those of chromium on the hepatic, renal, and respiratory systems (Wedeen and Qian, 1991). Of the organ system toxicities that excessive metal exposure can manifest itself with, it is perhaps the nervous system that is most sensitive. Metal neurotoxicity has been documented and studied for several metals, including copper (Cu), iron (Fe), manganese (Mn), mercury (Hg) and lead (Pb; Gutteridge, 1992; Stankiewicz *et al.*, 2007; White *et al.*, 2007; Wright and Baccarelli, 2007). Generally, the mechanism(s) of toxicity for these metals includes either binding to cellular proteins (i.e. Hg and Pb), or induction of oxidative stress within the exposed tissue (i.e. Cu, Fe, and Mn; Stankiewicz *et al.*, 2007; Wright and Baccarelli, 2007). It is the ability of transitions metals, like Mn, to promote oxidative stress within the nervous

system that has become an area of research, as CNS oxidative stress may be important in the initiation of neurological diseases such as Parkinson's Disease (Dobson *et al.*, 2004).

Essentiality, routes of exposure, and bioavailability of manganese

In modern times, Mn is encountered in mining and the smelting of metals, alloy industry, as a component of the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT), and in pesticides such as manganese ethylene bisdithiocarbamate (Maneb; Frumkin and Solomon, 1997; Aschner, 2000). Additionally, Mn is an essential dietary component and trace amounts are necessary for many physiological processes including synthesis of amino acids, lipids, proteins, and carbohydrates (Finley and Davis, 1999). Toxicologically, oral exposure to Mn is not a major route of exposure due to the low concentrations found in most foods and the limited transport by the gastrointestinal system (1-5%; Davidsson *et al.*, 1988; Davis *et al.*, 1993). Although parenteral nutrition may increase the exposure risk to Mn, typically this form of nutritional intake is only performed in controlled medical settings and on a limited basis (Wright and Baccarelli, 2007). Other potential routes of Mn exposure include the skin, a relatively impermeable barrier to charged particles, and the respiratory system. The absorption of Mn by the olfactory nerve and lungs is largely dependent on particle solubility ($MnSO_4$>Mn_3-$(PO_4)_4$) and this route of exposure, in terms of neurotoxicity, is toxicologically relevant (Roels *et al.*, 1997; Dorman *et al.*, 2001). Significant concentrations of Mn in the air can be found near industries involved in ferroalloy production, iron and steel foundries, and from power plant emissions (Lioy, 1983). Indeed, processing of Mn-containing ores

resulted in the first recorded Mn-caused neurotoxicity (reviewed by Aschner and Aschner, 1991).

Manganese toxicity and manganism

In 1837 ore grinders exposed to Mn dust exhibited extrapyramidal dysfunction with neuropsychiatric symptomatology referred to as "locura manganica" or "manganism" (reviewed by Aschner and Aschner, 1991). The overall effect of Mn on the central nervous system (CNS) is still not well understood. However, it has been demonstrated that Mn-induced neuropathology is similar to another neurological disorder of unknown etiology, Parkinson's Disease (PD). The study of this neurological disease has, in part, furthered our understanding of Mn neurotoxicity.

Parkinson's disease

In the early 19th century, the English physician James Parkinson described a shaking palsy in several patients (reviewed by Kempster *et al.*, 2007). This disease would later come to bear his name. Although little progress was made in understanding PD over the next 100 years, the Swedish neuroscientist, Arvid Carlsson, proposed in the 1960's that PD was the result of damage to the dopaminergic neurons in a region of the midbrain responsible for coordinating voluntary movement (Carlsson, 2001). This hypothesis was later shown to be correct and successful treatments were devised including the use of L-Dopa, one of the precursors necessary for the synthesis of dopamine (Carlsson, 2001). The study of PD has demonstrated the sensitivity of the dopamine-producing neurons to stressors, particularly oxidative stress (Dobson *et al.*, 2004; HaMai and Bondy, 2004).

In fact, as discussed below, it is oxidative stress that is thought to be partially responsible for the direct effects of Mn on dopaminergic neurons (Dobson *et al.*, 2004; HaMai and Bondy, 2004).

Mechanisms of manganese neurotoxicity

The sensitivity of dopaminergic neurons to Mn is partially attributed to the ability of Mn to interact with the catechols, such as dopamine and its precursors/metabolites, leading to their oxidation and generation of the reactive intermediates, and, ultimately, cell death (Graham *et al.*, 1978; Donaldson *et al.*, 1982; Archibald and Tyree, 1987; Sistrunk *et al.*, 2007). Alternatively, Mn has been shown to exert toxicity on neurons by directly disrupting neuronal mitochondrial respiration, leading to decreased ATP production and oxidative stress (Aschner and Aschner, 1991; Gavin *et al.*, 1999). This is supported by studies demonstrating that Mn-containing compounds, such as the fungicide Maneb and the fuel additive MMT, can inhibit mitochondrial respiration (Autissier *et al.*, 1977; Zhang *et al.*, 2003). Perturbation of mitochondrial function apparently is not limited to dopaminergic neurons as other neuronal types, e.g. striatal GABA interneurons are affected by Mn exposure (Liu *et al.*, 2006).

These effects are, in part, attributed to transport mechanisms that favor the influx, but not the efflux, of Mn into the mitochondria (Gunter *et al.*, 1994; HaMai and Bondy, 2004). Although research examining the ability of Mn, or other stressors, to directly damage neurons has been conducted, another potential target for Mn-induced neurotoxicity are two of the CNS glial cells, astroglia and microglia.

Inflammation and manganese

In the CNS, both astroglia and microglia are found in close proximity to neuronal cells. However, while astrocytes are uniformly distributed throughout the brain, microglial cells are not. In fact, areas of the brain affected by Mn, such as the striatum and the substantia nigra have more microglia than areas that appear to be less affected, i.e., brain stem and cerebellum (Lawson *et al.*, 1990; Savchenko *et al.*, 2000).

Recent publications have demonstrated the ability of Mn to stimulate, or augment, the ability of glial cells to produce inflammatory mediators that can damage surrounding neuronal cells. Astroglia, for example, accumulate Mn and can produce reactive oxygen species (ROS) and other substances that may be damaging to neurons (Aschner, 2000). Microglia are producers of several key inflammatory cytokines, such as TNF-α, and they are the major producers of this cytokine in the CNS (Kim and de Vellis, 2005). Moreover, as stated, it has been demonstrated that microglia and/or astroglia produce inflammatory mediators and microglia-derived mediators can stimulate astrocytes, thus creating a vicious inflammatory cycle. Such inflammatory cycle may be involved in the mechanisms of Mn neurotoxicity, especially in cases where an additional inflammatory stimulus leads to the activation of glial cells (Chang and Liu, 1999; Filipov *et al.*, 2005; Spranger *et al.*, 1998).

Due to the ability of microglial cells to process antigen for presentation to other cells of the immune system, as well as their ability to produce a diverse array of cytokines and immunological factors, microglia are considered the primary immune effector cells of the CNS (Perry *et al.*, 1998; Kreutzberg, 1996; Kim and de Vellis, 2005). Thus, it is possible that at least part of the Mn-induced neuronal toxicity is the result of

inappropriate, or inopportune, activation of the microglial cells, leading to enhanced and prolonged synthesis of inflammatory mediators and ensuing neuronal damage.

Activation of microglial cells may also occur as a consequence of neuronal cell damage or death. The release of neuronal cellular components like heat shock proteins and ATP have been shown to activate microglial cells (Kakimura *et al.*, 2002; Shimizu *et al.*, 2005; Potucek *et al.*, 2006; Light *et al.*, 2006). Alternatively, infection of the CNS by viral agents such as vesicular stomatitis virus and HIV has been shown to induce the (primarily) microglial production of inflammatory molecules such as nitric oxide (NO) and cytokines directly (Epstein and Gelbard, 1999). Additionally, systemic infection or peripheral inflammogen administration can activate CNS microglia, an effect of significant consequence in the presence of active neurodegeneration (Cunningham *et al.*, 2005), particularly in the aged (Godbout *et al.*, 2005). On the other hand, activation of microglia can occur in the absence of infection. Exposure to the model PD toxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is associated with microglial activation (McGeer *et al.*, 2003). Indeed, it is the potential for microglial activation by the previously listed mechanisms that explains the use of microglial activators, such as MPTP and bacterial lipopolysaccharide (LPS), in experimental models of PD or Mn neurotoxicity.

Microglia and Parkinson's Disease-like neuropathology

Early studies examining the role of microglia in PD-like neurotoxicity utilized the model PD toxicant MPTP. In both human and animal exposures to MPTP, prolonged activation of microglial cells was demonstrated long after exposure to MPTP had ended

(McGeer *et al.*, 2003). Additionally, it has been demonstrated that prior exposure to Mn before challenge with MPTP will result in greater basal ganglia pathology than exposure to Mn or MPTP alone (Takahashi *et al.*, 1989).

Since exposure to MPTP has been largely limited to i.v. drug users and laboratory animals, the relevance of this exposure model in the context of co-exposure to Mn becomes questionable. Although MPTP is a potent activator of microglia, a more relevant microglial activator may be lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria. LPS is a common environmental contaminant and model inflammogen due to its ability to stimulate microglia to produce cytokines, NO, and ROS (Chao *et al.*, 1992; Jeohn *et al.*, 2002b; Liu *et al.*, 2002). Indeed, binding of LPS to the recently discovered Toll-like Receptor 4 (TLR4) has been shown to be key for activation of cells of the innate immune system, including microglia (Bhat *et al.*, 1998; Jeohn *et al.*, 2002a; Medzhitov and Janeway, 1997; Takeda and Akira, 2004).

Toll-like receptors, intracellular signaling and inflammation

The discovery of the Toll-like receptor (TLR) family of proteins fundamentally changed the perceived role of the innate immune system. Interestingly, the initial finding that led to the discovery of the TLR family of proteins was not the result of experimentation in a typical animal model of innate immunity, rodent (mouse or rat), but in the developmental biology of the fruit fly, *Drosophila melanogaster*. Indeed, it was the discovery of the molecule Toll, which is required for the development of dorsal-ventral polarity in the fruit fly embryo, as well as fly immune responses to fungal pathogens, that led to the discovery of the TLR family of proteins (Anderson *et al.*, 1985;

Hashimoto *et al.*, 1988; Lemaitre *et al.*, 1996; Takeda and Akira, 2004; Beutler, 2004). Importantly, the discovery of the TLR molecules demonstrated that innate immune responses are not merely primitive and nonspecific, but are surprisingly complex and highly specific (Beutler, 2004; Takeda and Akira, 2004). Moreover, it has been suggested that TLR-mediated innate immunity is not only the first line of defense against invading pathogens, but that TLR are, possibly, the most important receptors in initiating both innate and adaptive immune responses (Beutler, 2004).

Although it is the recognition of pathogen-associated molecular patterns (PAMP) such as Gram negative bacterial LPS, viral RNA (polyinosinic:polycytidylic acid; pI:C), viral DNA (Cytosine Guanine linear dinucleotide; CpG), or Gram positive bacterial peptidoglycan (peptidoglycan from *Staphylococcus aureus*; GPG) by their TLR (TLR4, TLR3, TLR2, and TLR9, respectively) that initiate innate immunity and inflammation, it is the intracellular signal transduction pathways that control the expression/production of immune and inflammatory mediators (Beutler, 2004; Takeda and Akira, 2004). Generally speaking, TLR intracellular signaling is a series of activation events linking the TLR to adaptor molecules such as myeloid differentiation primary-response protein 88 (MyD88) or Toll-receptor-associated activator of interferon (TRIF), which subsequently activate primary kinases (Interleukin-1-receptor-associated kinase [IRAK], transforming growth factor-β activating kinase-1 [TAB1], TNF-receptor-associated factor 6 [TRAF6] or TRAF-family-member-associated NF-kB activator-binding kinase 1 [TBK1], and secondary signaling kinases Mitogen activated protein kinases; [MAPK]). Ultimately, these events lead to the activation of one or more transcription factors such as NF-kB, activator protein-1 (AP-1), or signal transducer and activator of transcription I (STAT I)

that induce inflammatory mediator mRNA expression and, in the case of inflammatory cytokines, subsequent protein production and release (Beutler, 2004; Takeda and Akira, 2004).

The specific signaling molecules induced by TLR-PAMP interaction are dependent on the TLR. For instance, TLR3-mediated intracellular signaling is independent of MyD88, instead requiring TRIF for initiation of intracellular signaling (Beutler, 2004; Kawai and Akira, 2006). In contrast, TLR2, TLR4, and TLR9 induce signaling via MyD88 (Beutler, 2004; Takeda and Akira, 2004). However, only TLR2 and TLR9 are entirely dependent on MyD88, as TLR4 can initiate intracellular signaling by both MyD88-dependent and MyD88-independent (i.e. TRIF) pathways (Beutler, 2004; Takeda and Akira, 2004). For MyD88-dependent signaling, activation of the primary kinases, including IRAK, TRAF6 and TAK1, leads to the activation of the MAPK and NF-kB, while TRIF (MyD88-independent) signaling activates TBK and IRF3, leading to the activation of the STAT I transcription factor (Beutler, 2004; Takeda and Akira, 2004).

Although TLR-induced intracellular signaling has been examined for several TLRs, the key role of TLR in both cellular innate immunity and inflammation is perhaps best illustrated by the first TLR-PAMP to be described, TLR4-LPS. Indeed, it was the observation that C3H/HeJ and C57BL/10ScCr mouse strains are resistant to LPS-induced endotoxic shock, but susceptible to gram negative bacterial infection (e.g. LPS-containing bacteria), that led to the discovery of TLR4 as the receptor for LPS (Poltorak *et al.*, 1998). Typically, binding of LPS to TLR4 leads to the association of the MyD88 adaptor protein (Figure 1-1). Subsequently, MyD88 recruits IRAK-1 and IRAK-4, serine-threonine kinases that phosphorylate TRAF6 (Beutler, 2004; Figure 1-1).

Activation of TRAF6 by IRAK-1/4 leads to the activation of the MAP3K TAK-1, leading to the activation of the MAPK cascade (Beutler, 2004; Takeda and Akira, 2004; Figure 1-1).

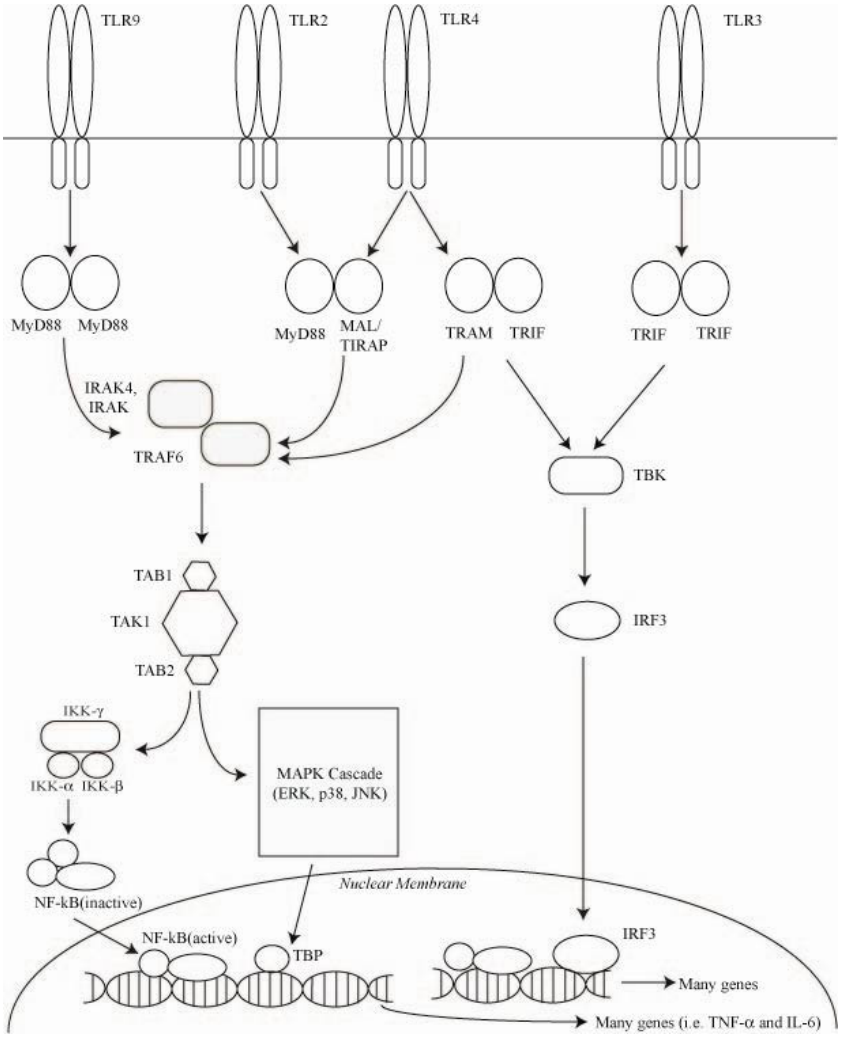

Figure 1-1 LPS signaling via the TLR4 receptor.

Figured adapted from Beutler (2004). Abbreviations: TLR (Toll-like receptors), MyD88 (myeloid differentiation factor 88), Mal/TIRAP (MyD88-Toll/Interluekin-1 receptor region adaptor protein), TRAM (Toll-receptor-associated molecule), TRIF (Toll-receptor-associated activator of interferon), IRAK (Interleukin-1 receptor-associated receptor kinase), TRAF (TNF-receptor-associated factor), TAK1 (Transforming-growth-factor-β-activated kinase), TAB (TAK1-binding protein), TBK (TRAF-family-member-associated NF-kB activator-binding kinase), IRF3 (Interferon-regulatory factor 3), IKK- (Inhibitor of nuclear factor-kB-kinase complex), NF-kB (nuclear factor-kB), MAPK (Mitogen-activated protein kinase), ERK (Extracellular regulated kinase), JNK (c-JUN n-terminal kinase), TBP (TATA-binding protein).

Once activated, TAK-1 phosphorylates the MAP2K (i.e. MEK-1/2, MEK-3/6, and MEK-4) which phosphorylate the MAPK, resulting in the initiation of cellular processes, including gene transcription (Seger and Krebs, 1995; Koistinaho and Koistinaho, 2002; Figure 1-2). The MAPK are composed of the extracellular signal-regulated kinases (ERK), stress-activated or c-Jun N-terminal kinases (SAPK/JNK), big MAPK 1 (BMK1), and the p38 MAPK (Seger and Krebs, 1995). Regulation of the MAPK cascade occurs by activation by 'upstream' MAP2K, as well as dephosphorylation of the MAPK due to the actions of phosphatases, such as the dual specificity phosphatases (DUSP; Camps *et al.*, 2000). The DUSP dephosphorylate the MAPK at one or both phosphorylation sites, leading to the deactivation of the MAPK (Camps *et al.*, 2000). Of these MAPK, p38 MAPK (p38) and ERK appear to be primarily involved in the production of inflammatory mediators by microglia (Koistinaho and Koistinaho, 2002).

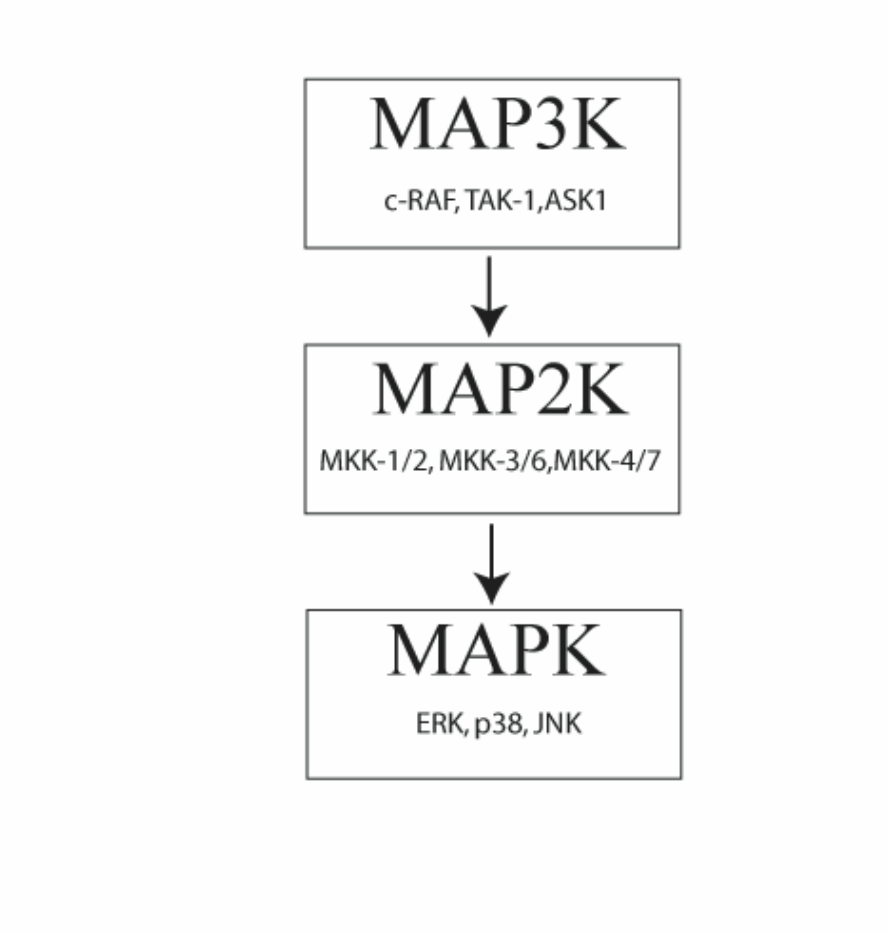

Figure 1-2 The MAPK Cascade

Microglial models of inflammation: mechanisms

In primary microglia and microglial cell lines, LPS has been shown to dose- and time-dependently increase the phosphorylation of ERK, JNK, and p38, as well as increase the expression of *i*NOS, TNF-α, and Cox-2 (Bhat *et al.*, 1998; Lee *et al.*, 1994; Lee *et al*., 1993; Waetzig *et al*., 2005). However, the p38-dependent increases in NO production require not only the phosphorylation of p38 but increased kinase activity as well (Jeohn *et al.*, 2002b).

Additionally, by exposing microglia to ERK- and p38-inhibitors prior to exposure to LPS, the LPS-induced increases in NO and TNF-α were inhibited (Bhat *et al.*, 1998). Furthermore, LPS-induced, p38-dependent, increases in NO and TNF-α by microglia have been shown to decrease neuronal survivability in neuronal-glial co-culture, an effect that can be inhibited by pretreatment with inhibitors of p38 (Jeohn *et al.*, 2002b). However, TLR expression and inflammatory cytokine production by microglia is not limited to TLR4, as microglia express mRNA for TLR 1-9 (Olson and Miller, 2004; Jack *et al.*, 2005), with 2, 3, 4, and 9 being the most abundant (Olson and Miller, 2004; Jack *et al.*, 2005). Furthermore, microglia produce IL-6 and TNF-α, as well upregulate cell-surface molecules such as major histocompatibility complex-II and costimulatory molecules including CD-40, CD-45, and CD-11b (Olson and Miller, 2004).

Manganese, microglia, and inflammation

Although inflammatory responses are essential for the maintenance and defense of tissues, uncontrolled or chronic inflammation can be detrimental to tissue homeostasis, especially in sensitive tissues such as the nervous system. In fact, abnormally high levels

of inflammatory cytokines, such as TNF-α, have been implicated in the etiology of PD (Nagatsu *et al.*, 2000). Within the context of Mn neurotoxicity, Mn enhances the production of inflammatory mediators by microglia, as exposure to Mn potentiates LPS-induced production of TNF-α, IL-6 (Filipov *et al.*, 2005) and NO (Chang and Liu, 1999; Filipov *et al.*, 2005) *in vitro*. Additionally, this effect is NF-kB-dependent as inhibitors of NF-kB were able to prevent the potentiation observed in Mn/LPS exposed cells (Filipov *et al.*, 2005).

Significance of research and hypothesis

In previous research, we have demonstrated that Mn-potentiated, LPS-induced inflammatory cytokine production by N9 microglia is NF-κB-dependent (Filipov *et al.*, 2005). At present, it is not known whether the potentiation of inflammatory cytokines and NO production by Mn occurs at the level of NF-kB or further upstream in the intracellular signaling cascade. Since potential 'upstream' targets include the MAPK family of kinases, it is reasonable to suggest that Mn may act on one, or more, of the MAPK. Moreover, as the MAP2K are responsible for activating the MAPK, while phosphatases (i.e. DUSP) deactivate MAPK, it is necessary to examine the role of these molecules in the potential activation of the MAPK by Mn exposure. Furthermore, as TLR intracellular signaling is differential, examining Mn-potentiation of microglial output of inflammatory mediators in N9 microglia activated by different TLR may provide additional data regarding the mechanism of Mn-potentiation. We hypothesize (i) that Mn enhances inflammatory cytokine production by acting on one or more of the MAPK molecules, leading to increased phosphorylation and prolonged activation of the

MAPK, (ii) if enhanced MAPK activation is observed, either increased MAP2K activation or decreased phosphatase activity may be involved, and (iii) enhanced MAPK activation may not be specific to TLR4 activation, as Mn may potentiate inflammatory cytokine production in microglia activated by other TLR.

CHAPTER II

MANGANESE POTENTIATION OF *IN VITRO* PROFINFLAMMATORY CYTOKINE PRODUCTION BY ACTIVATED MICROGLIAL CELLS IS ASSOCIATED WITH PERSISTENT ACTIVATION OF P38 MAPK[1]

Abstract

Previous studies that investigated the role of inflammation in the neurotoxicity of manganese (Mn) found that Mn enhanced the production of inflammogen (lipopolysaccharide; LPS)-induced inflammatory cytokines such as IL-6 and TNF-α. Although we have shown that the enhanced cytokine production occurs via a NF-κB-dependent mechanism, the role of upstream kinases in this Mn-induced enhancement has not been explored. As other studies have demonstrated that p38 mitogen activated protein kinase (p38) is necessary for LPS-induced, NF-κB-dependent expression of inflammatory cytokines, we hypothesized that Mn enhancement of LPS-induced production of IL-6 and TNF-α may be associated with p38 and conducted a series of experiments to address our hypothesis. We found that pre-treatment of microglial cells with a p38-inhibitor (SB203580) prevented Mn+LPS- induced production of IL-6 and

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1 Reprint with permission (Appendix A) from Crittenden, P. L., and Filipov, N. M. (2008). Manganese-induced potentiation of in vitro proinflammatory cytokine production by activated microglial cells is associated with persistent activation of p38 MAPK. *Toxicol In Vitro* **22**, 18-27.

TNF-α. Moreover, potentiation of IL-6 and TNF-α production, which occurred in both concurrent and sequential (3 h apart) exposures to Mn and LPS, was inhibited by inhibition of p38. Additionally, Mn exposure enhanced the phosphorylation and activity of p38 and this effect was persistent. Although p38 activity declined over time in vehicle and LPS-exposed cells, it persisted in cells exposed to Mn or Mn+LPS. Thus, the increased production of inflammatory cytokines by LPS-activated microglia exposed to Mn is associated with increased and persistent activation of p38.

Introduction

Manganese (Mn), while an essential metal, is also a common environmental contaminant. The presence of Mn in alloys, fertilizers, batteries, and fungicides, as well as the re-introduction of the fuel additive methylcyclopentdienyl manganese tricarbonyl (MMT), is of environmental and occupational concern (Frumkin and Solomon, 1997; Aschner, 2000). Occupational exposure to Mn has been linked to a specific neuropathology, manganism, that is characterized by clinical signs and lesions similar to Parkinson's Disease (PD; Meco *et al.*, 1994). Manganese is thought to exert its effects, at least partially, by disrupting mitochondrial respiration leading to increased oxidative stress (Aschner and Aschner, 1991; Gavin *et al.*, 1999). This is supported by studies demonstrating that Mn-containing compounds, such as the fungicide Maneb and the fuel additive MMT, can inhibit mitochondrial respiration (Autissier *et al.*, 1977; Zhang *et al.*, 2003).

While Mn is directly toxic to neuronal cells, neurons are not the only CNS cells that are associated with and contribute to Mn neurotoxicity. Astrocytes, for example,

accumulate Mn and may produce reactive oxygen species (ROS) and other substances that may be damaging to neurons (Aschner, 2000). Importantly, it has been demonstrated that the other CNS resident cells, the microglia, and/or the astrocytes may produce inflammatory mediators that could be involved in the mechanisms of Mn neurotoxicity, especially in cases where an additional inflammatory stimulus is present (Spranger *et al.*, 1998; Chang and Liu, 1999; Filipov *et al.*, 2005).

Microglia have been implicated in PD (humans and animal models) and research utilizing the model PD toxicant MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has shown that activated microglia persist long after exposure to MPTP has ended (McGeer *et al.*, 1987; McGeer *et al.*, 2003). Additionally, it has been demonstrated that prior exposure to Mn before challenge with MPTP will result in greater basal ganglia pathology than exposure to Mn or MPTP alone (Takahashi *et al.*, 1989). Therefore, this study suggests that Mn exposure has the potential of interacting with other basal ganglia toxicants, thus causing greater neurotoxicity. However, the likelihood of such exposure (*i.e.* Mn + MPTP) is remote. A more relevant model, however, may involve Mn and lipopolysaccharide (LPS). LPS is a common environmental contaminant (Niehaus and Lange, 2003) and model inflammogen due to its ability to stimulate microglia to produce cytokines, nitric oxide (NO), and ROS (Chao *et al.*, 1992; Jeohn *et al.*, 2002b; Liu *et al.*, 2002). Importantly, LPS exposure is also associated with basal ganglia toxicity (Niehaus and Lange, 2003) and it has been used as a model for PD (Castano *et al.*, 2002; Liu *et al.*, 2002)

Binding of LPS to CD14 and TLR4 cell surface receptors leads to the activation of intracellular kinases, including the mitogen activated protein kinases (MAPK; Bhat *et*

al., 1998; Jeohn *et al.*, 2002b). The MAPK family of proteins is comprised of the extracellular signal-regulated kinases (ERK), stress-activated or c-Jun N-terminal kinases (SAPK/JNK), big MAPK 1 (BMK1), and the p38 MAPK (Koistinaho and Koistinaho, 2002). Of these MAPK, p38 MAPK (p38) and ERK appear to be primarily involved in the production of inflammatory mediators by microglia. In primary microglia and microglial cell lines, LPS has been shown to dose- and time-dependently increase the phosphorylation of ERK and p38, as well as increase the expression of iNOS and TNF-α (Lee *et al.*, 1993; Lee *et al.*, 1994; Bhat *et al.*, 1998). However, the p38-dependent increases in NO production require not only the phosphorylation of p38 but increased kinase activity as well (Jeohn *et al.*, 2002a). Additionally, by exposing microglia to ERK- and p38-inhibitors prior to exposure to LPS, the LPS-induced increases in NO and TNF-α were inhibited (Bhat *et al.*, 1998). Furthermore, LPS-induced, p38-dependent, increases in NO and TNF-α by microglia have been shown to decrease neuronal survivability in neuronal-glial co-culture (Jeohn *et al.*, 2002a; Jeohn *et al.*, 2002b). The fact that this effect can be inhibited by pretreatment with inhibitors of p38 suggests that p38 appears to play a dominant role in the process.

Although inflammatory responses are essential for the maintenance and defense of tissues, uncontrolled or chronic inflammation can be detrimental to tissue homeostasis, especially in sensitive tissues like the nervous system. In fact, abnormally high levels of inflammatory cytokines, such as TNF-α, have been implicated in the etiology of PD (Nagatsu *et al.*, 2000). Within the context of Mn neurotoxicity, Mn enhances the production of inflammatory mediators by microglia. Indeed, exposure to Mn potentiates LPS-induced production of inflammatory cytokines (TNF-α & IL-6) and NO *in vitro*

(Filipov *et al.*, 2005). Additionally, this effect is NF-kB-dependent as inhibitors of NF-kB were able to prevent the potentiation observed in Mn+LPS exposed cells (Filipov *et al.*, 2005). At present, it is not known whether the potentiation of inflammatory cytokine production by Mn occurs at the level of NF-kB or further upstream in the intracellular signaling cascade. Since potential upstream targets include p38 and ERK and because a p38 inhibitor alone or in combination with an ERK inhibitor prevents the LPS-induced production of inflammatory mediators (Bhat *et al.*, 1998), we conducted preliminary studies examining the effect of MAPK inhibition on cytokine production in Mn-exposed microglial cells activated with LPS (Crittenden and Filipov, 2004). From these studies we determined that inhibition of p38, but not of ERK, eliminated the potentiation of LPS-induced cytokine production in N9 microglial cells.

After we established the role of p38 in the enhancement of LPS-induced inflammatory cytokine production by Mn, our objectives in this study were to (i) examine in detail the functional activation of p38 by exposure to Mn by itself or in combination with LPS (ii) to evaluate the time-window during the inflammatory cytokine production process which is dependent upon p38 in Mn+LPS-exposed microglia**.**

Materials and Methods

Chemicals. Unless specified, all chemicals and reagents were purchased from Sigma-Aldrich (Sigma; St. Louis, MO) and $MnCl_2$ with purity >99% was used.

Cell Culture. The N9 murine microglial cell line used in the experiments was a gift kindly provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy). These

cells, derived by a retroviral immortalization of day-13 embryonic mouse brain cultures, are similar to primary microglia in that, upon activation, they produce inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, as well as NO (Righi *et al.*, 1989).

The cultures were maintained (5% CO_2, 95% air, at 37°C) in RPMI-1640 supplemented with 10% FBS, 0.075% sodium bicarbonate, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 25 μg/ml gentamycin, 100 U/ml penicillin G, and 100 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA). For pharmacological inhibition and cytokine studies, cells were seeded at 0.25 x 10^6 cells/well (0.5 ml volume) in 48-well plates (Costar; Fisher Scientific, Pittsburgh, PA). For western blot protein analysis, cells were seeded at 2.5 x 10^6 cells/well (5 ml volume) in 6-well plates (Costar), whereas for the flow cytometry analysis, the density was 2.0 x 10^6 cells/well (4 ml volume). Cells were incubated for up to 24 h in the presence of Mn (250 μM) and/or LPS (*Escherichia coli* 055:B5; Calbiochem, La Jolla, CA; 100 ng/ml).

In our preliminary studies (Crittenden and Filipov, 2004), and in Filipov *et al*., (2005), Mn concentrations of 50-1000 μM and LPS of 10-1000 ng/ml were used, and the potentiation of cytokine production was both Mn- and LPS-dependent. Hence, the concentrations of Mn and LPS used in this study were selected based on previous experiments that indicated no significant N9 cell death following exposure to Mn and/or LPS at these concentrations (Filipov *et al.*, 2005) and are in line with numerous other *in vitro* studies where levels of Mn (Mn^{2+}) range from 10 μM to 4 mM with the most typical exposure range being 100 to 500 μM (Li *et al.*, 2005; Malthankar *et al.*, 2004).

Moreover, the above concentrations and the concentrations used in our study are representative of Mn levels found in brains of non-human primates following exposure to manganese dioxide for 3 months (ranging from 35 to 350 μM; Suzuki *et al.*, 1975). Elevated levels of Mn in the basal ganglia are not associated only with occupational exposure, but have been also observed in the brains of PD patients, as well as in autopsied brains of patients with cirrhosis (Yase, 1972). Of note, the concentration of LPS we have used in the present study (100 ng/ml) is relatively low and we have already reported that when greater amounts of LPS are present in the culture medium, less Mn is required for a potentiating effect on cytokine production to be observed (Filipov *et al.*, 2005).

Cytokine Analysis. The amounts of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the supernatants were determined using DuoSet ELISA kits (R&D; Systems, Inc., Minneapolis, MN) as per manufacturer's instructions. Briefly, 96-well microplates (Costar) were coated with 1:180 dilution of respective capture antibody in PBS and incubated overnight at room temperature (RT). The plates were then washed with PBS-Tween buffer (3x) and blocked (1% BSA, 5% sucrose in PBS) for 1 h at RT. Next, plates were washed again (3x) and either samples or standards were dispensed into the wells. After incubating for 2 h at RT the plates were washed (3x), incubated with detection antibody for 2 h, and washed again (3x). Strepavidin-HRP conjugate was then added for 20 min, plates were washed (3x), and TMB-based substrate was added to each well and allowed to develop up to 30 min. The reaction was stopped by addition of 50 μl of 2N H_2SO_4 per well and the absorbance was read using a Spectramax Plate Reader

(Molecular Devices, Sunnyvale, CA) at 450 nm. All samples for each cytokine were assayed in duplicate, and the mean was used in the subsequent statistical analysis. A standard curve was generated for each plate.

Pharmacological manipulations. The p38 inhibitor, SB203580 (Calbiochem, San Diego, CA), was added 3 h prior to, or after, the addition of Mn, LPS, or Mn and LPS to the cell culture. The inhibitor was dissolved in DMSO (26.5 mM) and stored at –80°C until diluted to a working concentration (50 μM) prior to use. The final DMSO concentration was < 0.2% for vehicle and p38-inhibitor exposed cells.

Immunoblot analysis of p38 and phospho-p38. After incubation for 15 min, 1 and 4 h, cells were removed from the culture well via scraping and the cell suspension was centrifuged (300g; 10 min; 4°C). Following centrifugation, the supernatants were discarded, cells were resuspended in 100μl of RIPA (modified radioimmuno-precipitation) lysis buffer (1x PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing PMSF (Sigma), protease and phosphatase inhibitors (Protease Inhibitor Cocktail, Sigma, and Halt Phosphatase Inhibitor Cocktail, Pierce [Rockford, IL], respectively), and held on ice for 30 min with occasional pipetting to disrupt cell membranes. Protein concentration in the cell lysates was determined using the Bradford method with reagents obtained from Bio-Rad (Hercules, CA) with BSA as a standard. Aliquots of each sample were diluted in reducing sample buffer and heat denatured for 5 min at 95 ºC. Twenty micrograms of total protein were loaded and separated on a 10% SDS-PAGE gel, transferred to a PVDF membrane, and the membranes were blocked in 5% milk for 1 h at RT. The membranes were then incubated overnight with rabbit

antibodies specific for the non-phosphorylated or phosphorylated p38 (Cell Signaling Technology, Beverly, MA) at 1:1000 and 1:500 dilutions, respectively, washed 3x, and then probed with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-Rad) at a 1:10,000 dilution. The blots were exposed to SuperSignal West Pico chemiluminescent substrate (Pierce) for up to 5 min and then enclosed in transparent covers prior to exposure to x-ray film. Band density was analyzed using the UN-SCAN-IT software package (Silk Scientific Inc., Orem, UH).

Phospho-p38 analysis by flow cytometry. Four ml of 0.5 x 10^6 cells/ml N9 cell suspension (2 x10^6 cells/well) were plated overnight in 6-well plates as described above. Cells were incubated with Mn/LPS for 15 min, 1, and 4 h. Following incubation, cells were lifted from the culture wells with a cell lifter, spun down (10 min; 300 xg; 4 ºC), and resuspended in 0.5 ml of PBS. Cells were then fixed by adding of 0.5 ml of 4% paraformaldehyde (2% final concentration) to the tubes and incubating them for 10 min at 37ºC. Next, the cells were chilled for 1 min on ice, centrifuged (10 min; 300 xg; 4 ºC), and permeabilized by resuspending them in 1 ml of 90% methanol and an incubation for 30 min on ice, followed by an overnight incubation at -20 ºC. The next day, cells were centrifuged to remove the methanol and washed twice with a binding buffer (1% BSA in PBS, 2 ml for each wash). Next, 1 ml of the cell suspension (approximately 0.6 x 10^6 cells) was transferred to flow tubes, spun down, and resuspended in 100 µl of binding buffer. Following 10 min incubation at RT, 10 µl/tube of the anti-phospho-p38 (T180/Y182)-PE pre-titrated antibody, or an appropriate PE-conjugated isotype control (mouse IgG_1; both from BD Biosciences, San Diego, CA) were added and the tubes

incubated for 60 min at RT in the dark. At the end of this incubation, the cells were washed twice with a binding buffer, resuspended in 0.5 ml of PBS and analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

p38 activity analysis. The enzymatic activity of the phosphorylated p38 was determined by a western blot analysis using the p38 MAP Kinase Assay Kit (Cell Signaling Technology). Briefly, following exposure to Mn and/or LPS the cells were rinsed once with ice-cold PBS followed by incubation with 0.5 ml of ice-cold 1x lysis buffer containing 1mM PMSF for 5 min. Cells were scraped and transferred to 1.5 ml tubes, sonicated 4x for 5 sec, and centrifuged (14,000 x g) for 10 min at 4 ºC. Next, 20 µl of bead-immobilized antibody slurry was added to 200 µl of sample supernatant and incubated overnight at 4 ºC. After centrifugation (14,000 x g) for 10 min at 4 ºC), the samples were washed (2x) in 1x lysis buffer followed by 2 washes in 1x kinase buffer. Finally, each sample was resuspended in 50 µl of 1x kinase buffer supplemented with 200 µM ATP and 2 µg of kinase substrate (ATF-2 fusion protein). All samples were incubated for 30 min at 30 ºC. The kinase reaction was terminated by denaturing at 95 °C for 5 min in 3x SDS sample buffer containing 150 mM DTT. The samples were electrophoretically separated (10% SDS-PAGE gel) and transferred to PVDF membranes. The membranes were blocked in 5% w/v nonfat milk and probed with a polyclonal, rabbit IgG anti-Phospho-ATF-2 (Thr71) antibody at a 1:2000 dilution. Following washing (3X), the membranes were incubated with HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-biotin antibody (for biotinylated protein molecular weight markers). The membranes were incubated for 1 min in LumiGLO substrate and wrapped in sheet

protectors. The membranes were exposed to X-ray film for 10 s, the films developed and band density analyzed using the UN-SCAN-IT software package.

Statistical analysis. Data were analyzed using analysis of variance (ANOVA). When statistical differences were detected ($P < 0.05$), treatment means were separated by the Student Newman Keuls (SNK) *post hoc* test. All data are presented as means $\pm$ S.E.M.

Results

Pharmacological manipulation. To determine the time-dependency in the ability of Mn to enhance LPS-induced pro-inflammatory cytokine production and its association with p38, in addition to exposing cells to Mn+LPS as before (Filipov *et al.*, 2005), we exposed cells to 250 μM Mn 3 h before or after activation by 100 ng/ml LPS. As previously observed, exposure to Mn+LPS potentiated the microglial production of TNF-α (Figure 2.1A) and IL-6 (Figure 2.1B). Cells activated with LPS 3 h before or after exposure to 250 μM Mn produced more TNF-α (Figure 2.1A) and IL-6 (Figure 2.1B) in comparison to LPS-alone. In terms of the timing of exposure to Mn or LPS, the observed potentiation effect was the lowest when Mn was added 3 h after LPS, but, nevertheless, the potentiation was still present.

Exposure to 250 μM Mn and 50 μM SB203580 3 h after LPS activation prevented Mn-induced potentiation of TNF-α and IL-6 (Figures 2.1A and 2.1B). Similarly, exposure to Mn followed by SB203580 and LPS 3 h later also prevented Mn-induced potentiation of TNF-α and IL-6 (Figures 2.1A and 2.1B, respectively). Additionally,

SB203580 inhibited cytokine production in cells exposed simultaneously to Mn+LPS, regardless of whether Mn+LPS exposure occurred before, or after, SB203580 (Figures 2.1A and 2.1B).

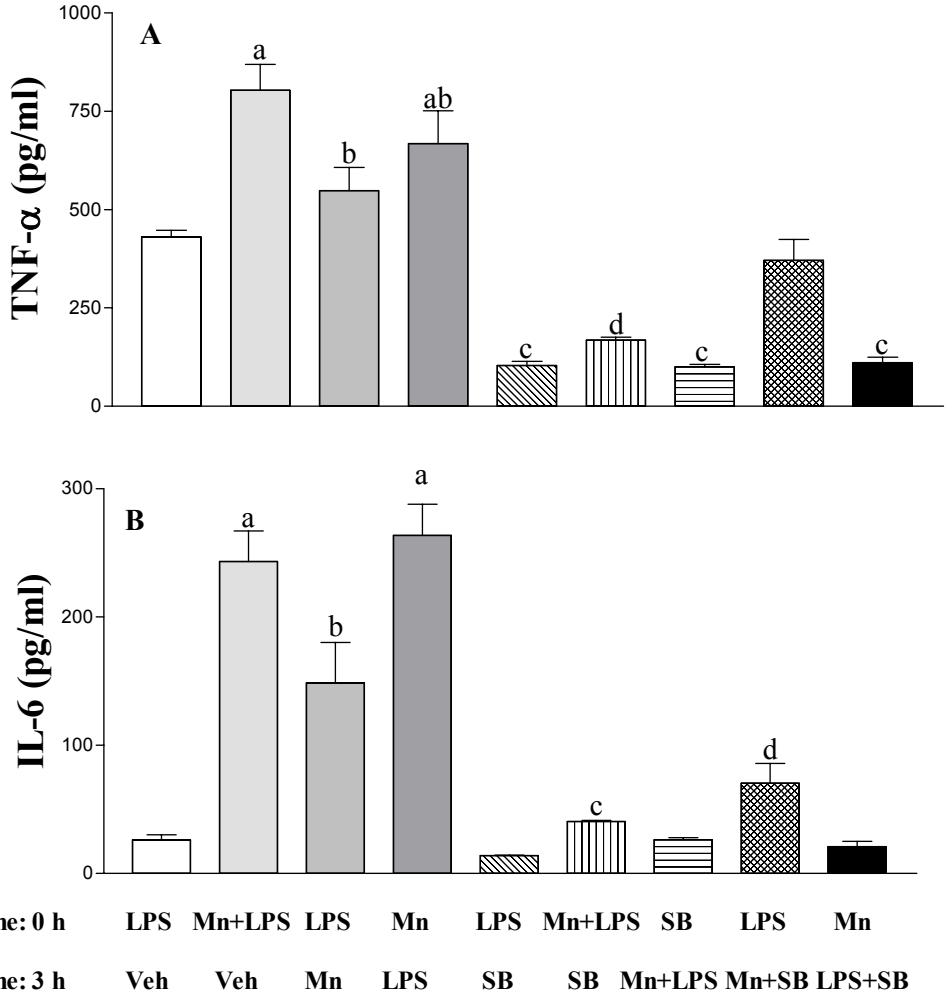

Figure 2.1 Effect of delayed exposure to Mn and/or SB203580 (a p38 inhibitor) on LPS-activated microglial cytokine production.

TNF-α (1A) and IL-6 (1B) production was analyzed in supernatants collected 24 h after exposure of N9 microglial cells to LPS (100 ng/ml) then Vehicle (3 h later), Mn (250 μM) + LPS then Vehicle (3 h later), LPS then Mn (3 h later), Mn then LPS (3 h later), LPS then SB203580 (3 h later), LPS then Mn + SB203580 (50 μM), Mn then LPS + SB203580, Mn + LPS then SB203580, and SB203580 then Mn + LPS. Media levels of TNF-α and IL-6 were analyzed by respective DuoSet ELISA as described in the Materials and Methods. Data shown in each bar represent the mean ± S.E.M. of 4-8 independent replicates. [a, b, c, d] Presence of different letters indicate bars are significantly different from control and previous exposures ($P < 0.05$).

Immunoblot analysis of p38 and phospho-p38 protein levels. To examine p38 activation, we studied the induction of p38 phosphorylation following exposure to Mn (250 μM) with and without LPS (100 ng/ml) for up to four hours after exposure. The levels of non-phosphorylated p38 (all exposures) did not change in response to Mn and/or LPS exposure (data not shown). However, induction of p38 phosphorylation (normalized to non-phosphorylated p38) was observed as early as 15 min following Mn and/or LPS exposure (Figure 2.2A). Increased phosphorylation was observed at 1 h for Mn+LPS and this effect persisted through the 4 h time point (Figures 2.2B and 2.2C, respectively). On the other hand, the LPS-induced phosphorylation had almost returned to control levels, while Mn-alone increased p38 phosphorylation at the 4 h time point (Figure 2.2C).

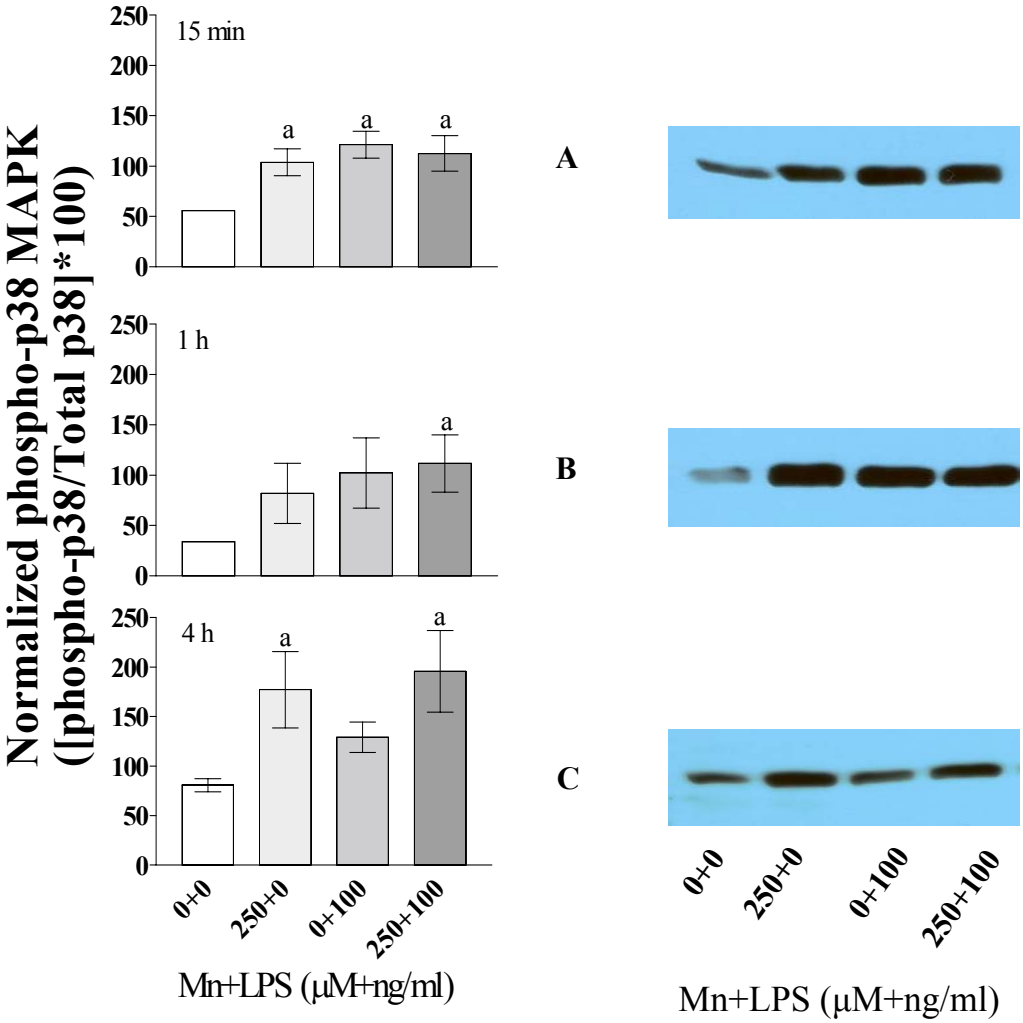

Figure 2.2 Densitometric analysis (left panels) and representative western blots (right panels) of phosphorylated p38 kinase in N9 microglia exposed to Mn, LPS, or Mn+LPS.

(A) Phosphorylated p38 protein from N9 cells exposed to vehicle, 250 µM Mn, 100 ng/ml LPS, or 250 µM Mn+100 ng/ml LPS for 15 min (A), 1 h (B) and 4 h (C). Western blots and densitometric analyses were performed as described in the Materials and Methods. Data shown in each bar represent the mean ± S.E.M. of the adjusted pixel density for phosphorylated p38 normalized to non-phosphorylated p38. Each bar represents a minimum of 3 independent replicates. [a] Presence of letters indicate treatment differences within a time point ($P < 0.05$).

Phospho-p38 analysis by Flow Cytometry. Similar to the western blot data (Figure 2.2), flow cytometry results indicated a time-dependent effect of LPS, Mn, and Mn+LPS on phospho-p38 (Figure 2.3). Thus, in the absence of Mn, 100 ng/ml LPS-induced increase was rapid (15 min), but it plateaued at 1 h and begun to subside at the 4 h time point. On the other hand, 250 μM Mn increased the phospho-p38 levels by 15 min in comparison with the effect being significant at the 1 and 4 h time points. While at 15 min the Mn+LPS combination increased phospho-p38 to an extent similar to LPS, at the 1 and 4 h time points, the Mn+LPS effects were greater than the effects of either Mn or LPS alone. Importantly, similar to the western blot data, by 4 h the LPS-induced phosphorylation had returned to near control levels.

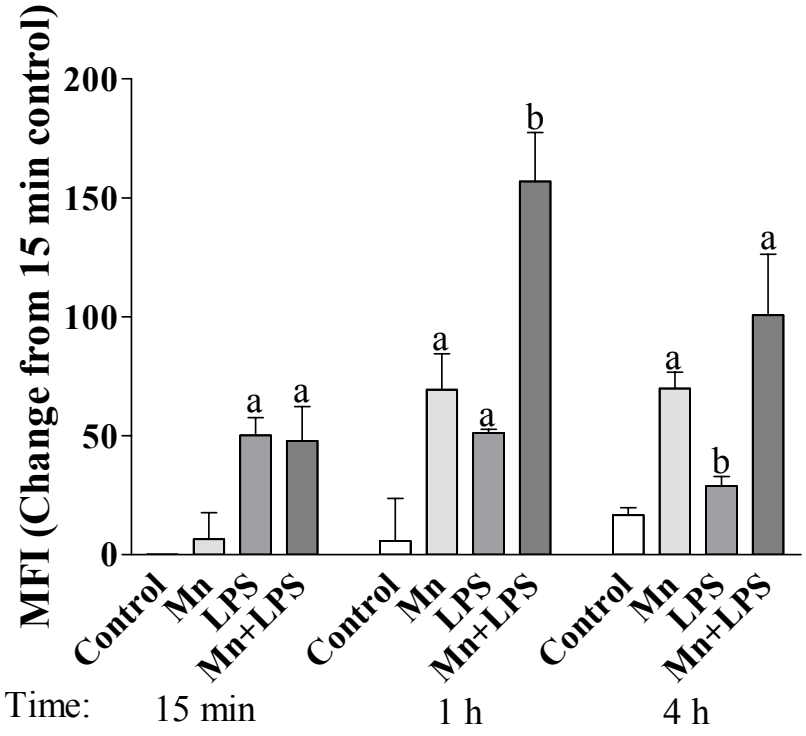

Figure 2.3 Phosphorylated p38 protein levels in N9 microglia exposed to 250 μM with or without 100 ng/ml LPS.

Cells collected after 15 min, 1 h, or 4 h exposure were analyzed by flow cytometry as described in the Materials and Methods. Data shown represent the mean fluorescence intensity (MFI) change from the 15 min control ± S.E.M. for two independent experiments where each experimental condition was independently replicated (n =4). [a, b] Presence and different letters indicate treatment differences within a time point ($P < 0.05$).

p38 activity. The kinase activity of p38 was measured using western blot detection of a phosphorylated-substrate (ATF-2 Fusion Protein). Exposure to 250 μM Mn, 100 ng/ml LPS, or Mn+LPS increased p38-kinase activity at 15 min and 4 h compared to control (Figures 2.4A, and 2.4B). This effect persisted through the 4 h time point, but only for the Mn and Mn+LPS exposed cells (Figure 2.4C).

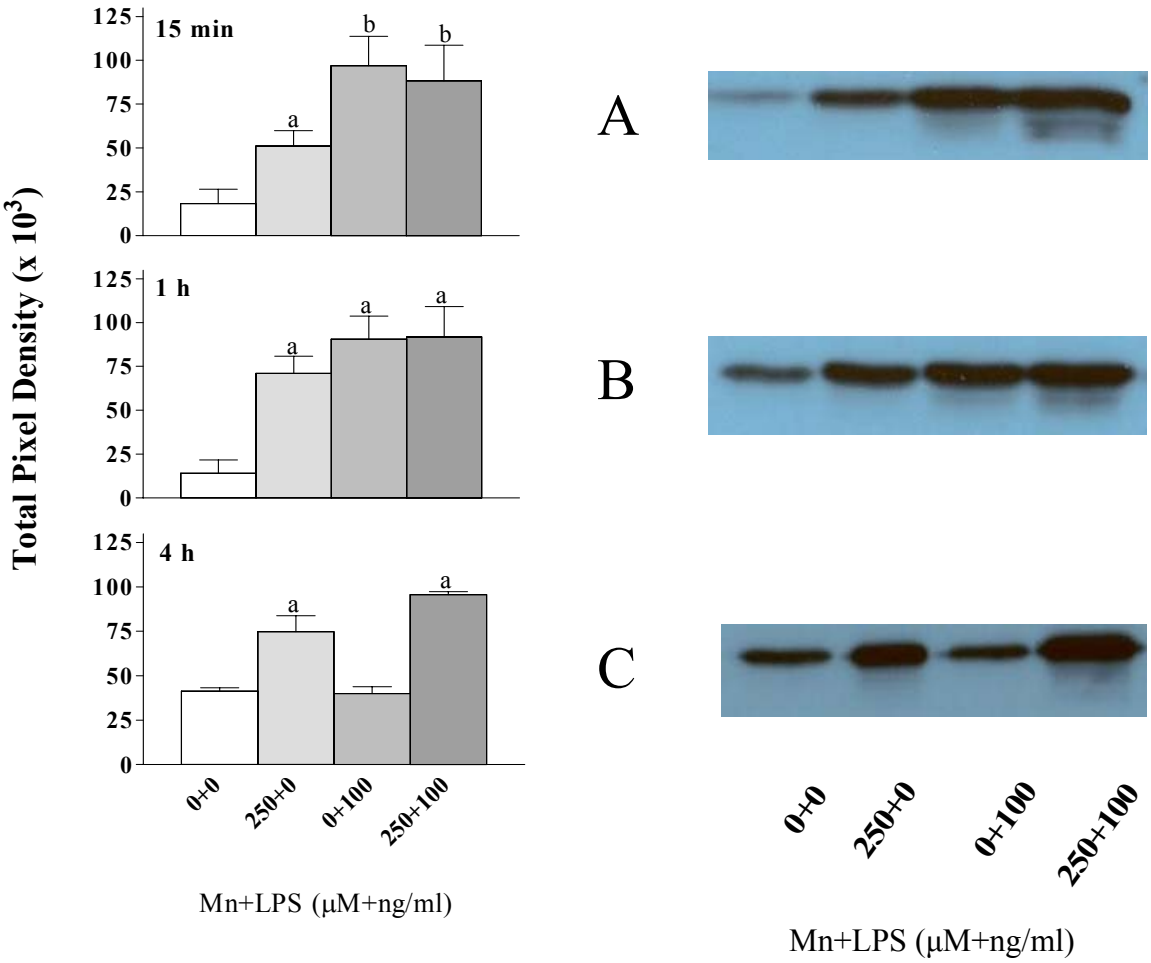

Figure 2.4 p38 activity following exposure to vehicle, 250 μM Mn and/or 100 ng/ml LPS for 15 min (A), 1 h (B), or 4 h (C) in N9 microglia cells.

The p38 activity in whole cell lysates was analyzed by western blot for the detection of a phosphorylated p38 substrate (ATF-2 Fusion Protein) followed by densitometric analyses (left hand panel) as described in the Materials and Methods. Blots representative of three independent experiments are shown in the right hand panel. Data shown in each bar the mean ± S.E.M. of the total pixel density for 3 independent replicates. [a,b] Presence and different letters indicate treatment differences within a time point ($P < 0.05$).

Discussion

Although it has been demonstrated that microglia play a role in basal ganglia neurotoxicity (Olanow and Tatton, 1999; Castano *et al.*, 2002), how environmental toxicants like Mn contribute to microglial-mediated inflammation has only recently been explored. Brain regions that possess an abundance of microglial cells are especially sensitive to inflammatory stimuli, particularly the bacterial endotoxin LPS (Kim and de Vellis, 2005). Activation of macrophages and microglia by LPS is thought to occur (primarily) by binding of LPS to TLR4, leading to activation of intracellular kinases (i.e. MAPK) and transcription factors like NF-κB (Medzhitov and Janeway, 1997; Takeda and Akira, 2004). Moreover, activation of the MAPK and NF-κB by LPS results in the production of inflammatory mediators such as NO and TNF-α (Bhat *et al.*, 1998; Lee *et al.*, 1993; Lee *et al.*, 1994).

Previous research has demonstrated that Mn exposure, in combination with LPS, can increase the production of iNOS and NO by microglial cells (Chang and Liu, 1999; Filipov *et al.*, 2005; Chen *et al.*, 2006), as well as the production of the inflammatory cytokines TNF-α (Filipov *et al.*, 2005; Chen *et al.*, 2006) and IL-6 (Filipov *et al.*, 2005). On its own, Mn exposure increases the cytokine production only slightly and at higher concentrations, suggesting that microglial activation must be present in order for the Mn effects on TNF-α and IL-6 to be observed. In this study, similar to Filipov *et al*., (2005), we observed a strong potentiation of TNF-α and IL-6 production by N9 microglial cells following exposure to Mn+LPS compared to exposure to LPS alone. Interestingly, Mn-potentiation of IL-6 in activated microglia was greater than for TNF-α, with IL-6

concentrations increasing 5-fold or more, in comparison to 2-fold changes in TNF-α. On the other hand, exposure to the p38 inhibitor SB203580 inhibited TNF-α in Mn+LPS-exposed cells to a greater extent than the inhibition observed for IL-6, suggesting that although both cytokines depend on p38 for their synthesis, the degree of dependency may be different for the two cytokines

Importantly, we were also able to demonstrate that Mn can potentiate LPS-induced cytokine production if the Mn-exposure occurs 3 h after the exposure to the inflammatory stimulus, or, alternatively, that prior (3 h) exposure to Mn can potentiate the LPS-induced inflammatory cytokine production. To our knowledge, this is the first report of this observation, providing additional data to support the hypothesis that Mn exposure can potentiate the production of inflammatory cytokines *in vitro* and, possibly, inflammation *in vivo* not only in cases of co-exposure to an inflammogen and Mn, but also in cases where the exposure is, within a (possibly) narrow window, sequential.

LPS has been shown to activate the MAPK family of kinases, leading to the production of inflammatory mediators such as NO and TNF-α (Bhat *et al.*, 1998; Lee *et al.*, 1993; Lee *et al.*, 1994). While for microglia, both p38 and ERK MAPK have been shown to contribute to the production of inflammatory mediators (Bhat *et al.*, 1998; Jeohn *et al.*, 2002a; Koistinaho and Koistinaho, 2002; Zarubin and Han, 2005), p38 appears to play a dominant role. For example, LPS-stimulated production of NO and cytokines by microglia can be inhibited by prior exposure to a p38 inhibitor (Bhat *et al.*, 1998; Jeohn *et al.*, 2002a). This is also the case with Mn potentiation of LPS-induced cytokine expression in the present study, as exposure to SB203580 before or after exposure to Mn+LPS inhibited the production of IL-6 and TNF-α by N9 microglial cells.

In support, our western blot and flow cytometry p38 activity data indicated that the increased p38 activity caused by Mn persists for at least 4 h.

In contrast, the ERK inhibitor (PD98059) has a very small effect on IL-6 and TNF-α production by the Mn+LPS exposed microglia (Crittenden, 2004; data not shown), suggesting that activation of other MAPK which are known to contribute to the production of inflammatory mediators in microglia (Bhat *et al.*, 1998; Koistinaho and Koistinaho, 2002; Zarubin and Han, 2005), play only a minor role in the Mn+LPS-induced production of TNF-α and IL-6.

Two other recent studies have investigated the role of intracellular kinases in the Mn effects on NO/iNOS and/or TNF-α production using the microglial cell line BV-2 (Bae *et al.*, 2006) or primary mixed glial cells (Chen *et al.*, 2006). Bae *et al*. (2006) examined the effects of Mn (100 μM to 1 mM) by itself only on iNOS and suggested that multiple MAP kinases were phosphorylated by Mn and that, based on inhibitor data, the p38 kinase appeared not to play a major role in the Mn-induced iNOS. Nevertheless, even though not quantified, these authors did report that p38 phosphorylation was increased by Mn at 1, 3 and 6 h post exposure, thus agreeing with our data. In their studies with primary glial cells, which contain both astrocytes and microglia (85 and 15% , respectively), Chen *et al*. (2006) observed that (i) the phosphorylation of multiple (p38, ERK, JNK) kinases was induced by 30 min exposure to Mn, either by itself or in combination with LPS/IFN-γ, (ii) similar to our present data and to Filipov *et al*. (2005), while having no effect on its own, Mn potentiated TNF-α production by glial cells exposed to LPS/IFN-γ, and (iii) pharmacological inhibition of p38 was one of the ways of

inhibiting the potentiating effect of Mn on TNF-α production. Interestingly, these authors also suggested that iNOS/NO production may be regulated in a manner different from that of TNF-α. Importantly, similar to our present studies, Chen *et al.* (2006) indicated that an activation of MAPKs by Mn is not sufficient to trigger the glial cell production of TNF-α, but that it is critical for the potentiating effects of Mn on LPS/IFN-γ -stimulated TNF-α production to be observed.

Having observed that p38 was required for the Mn-potentiation of cytokine production, it was necessary to examine p38 phosphorylation directly as enhanced phosphorylation is associated with increased activity and thus could increase the production of IL-6 and TNF-α. Indeed, this observation was supported by western blot analysis and flow cytometry. Although LPS did increase the phosphorylation of p38 through the 1 h time point, by 4 h this effect had diminished. In contrast, prolonged phosphorylation of p38 was observed in Mn-exposed cells, persisting through the 4 h time point. In comparison, increased p38 activation was observed in Mn-exposed cells, an effect that persisted through the 4 h time point. LPS alone increased p38 activity, however by 4 h the LPS-induced increase in p38 activity had largely subsided. Moreover, we have observed enhanced p38 activation 24 h following exposure to Mn or Mn+LPS (data not shown).

While p38 is clearly associated with the Mn-potentiation of TNF-α and IL-6 by activated microglia, how Mn enhances the phosphorylation and activity of p38 is not apparent at this time. One possibility is that Mn acts at a site other than p38, perhaps upstream at the level of the MAPK kinase (MKK). The MKKs are responsible for phosphorylating MAPK to its active form. Two MKK that are known to activate p38 are

MKK3 and MKK6 (Koistinaho and Koistinaho, 2002; Zarubin and Han, 2005). Increased activity by MKK3 or MKK6 could lead to enhanced p38 MAPK activation (Enslen *et al.*, 1998). Alternatively, inhibition of the enzymes responsible for inactivating p38, the dual specificity phosphatases (DUSP; Groom *et al.*, 1996), may be responsible for the prolonged phosphorylation following exposure to Mn/LPS that was observed in the current study. In this regard, another metal, arsenic, has been shown to inhibit a different DUSP responsible for maintaining low basal levels of JNK (Cavigelli *et al.*, 1996). Additionally, it has been demonstrated that ERK can negatively regulate p38 via the MAPK phosphatase MKP-1 (Carter *et al.*, 1999). It is possible that Mn inhibits either ERK-mediated activation of MKP-1 or, MKP-1 activity.

Our earlier study indicated that the Mn-induced potentiation of IL-6 and TNF-α is NF-κB dependent (Filipov *et al.*, 2005). With our present study we demonstrate that p38 is critical for the Mn-induced potentiation of cytokine production to occur. Exactly how NF-κB and p38 are interacting in Mn+LPS exposed microglia is the subject of future studies. A possible mechanism involves NF-κB and regulation of gene transcription. Active p38 phosphorylates the TATA-binding protein (TBP), resulting in the association of TBP with the TATA-promoter as it has already been demonstrated that p38 phosphorylation of TBP is required for NF-κB-dependent gene expression, including TNF-α and IL-6 (Carter *et al.*, 1999). As Mn-potentiation of LPS-induced inflammatory cytokine production appears to require both p38 and NF-κB, it is possible that the observed increases in p38 activity may increase phosphorylation of TBP leading to enhanced NF-κB-dependent gene expression of IL-6, TNF-α and, possibly, other

inflammatory mediators. This is further supported by the observation in the present study that increased phosphorylation of p38 was observed in cells exposed to Mn in the absence of LPS. However, Mn exposure alone resulted in negligible amounts of IL-6 and TNF-α produced by the N9 microglia as demonstrated in our previous study (Filipov *et al.*, 2005). As synthesis of IL-6 and TNF-α is NF-κB-dependent, and exposure to LPS leads to activation of NF-κB, it is likely that Mn-potentiation of inflammatory cytokine production occurs via a p38-dependent mechanism, but requires the subsequent/concurrent activation of NF-κB. Considering the interactive nature of intracellular signaling, it is possible that Mn may be acting at multiple sites leading to increased p38 phosphorylation and activity and subsequent IL-6 and TNF-α production as proposed in Figure 2.5.

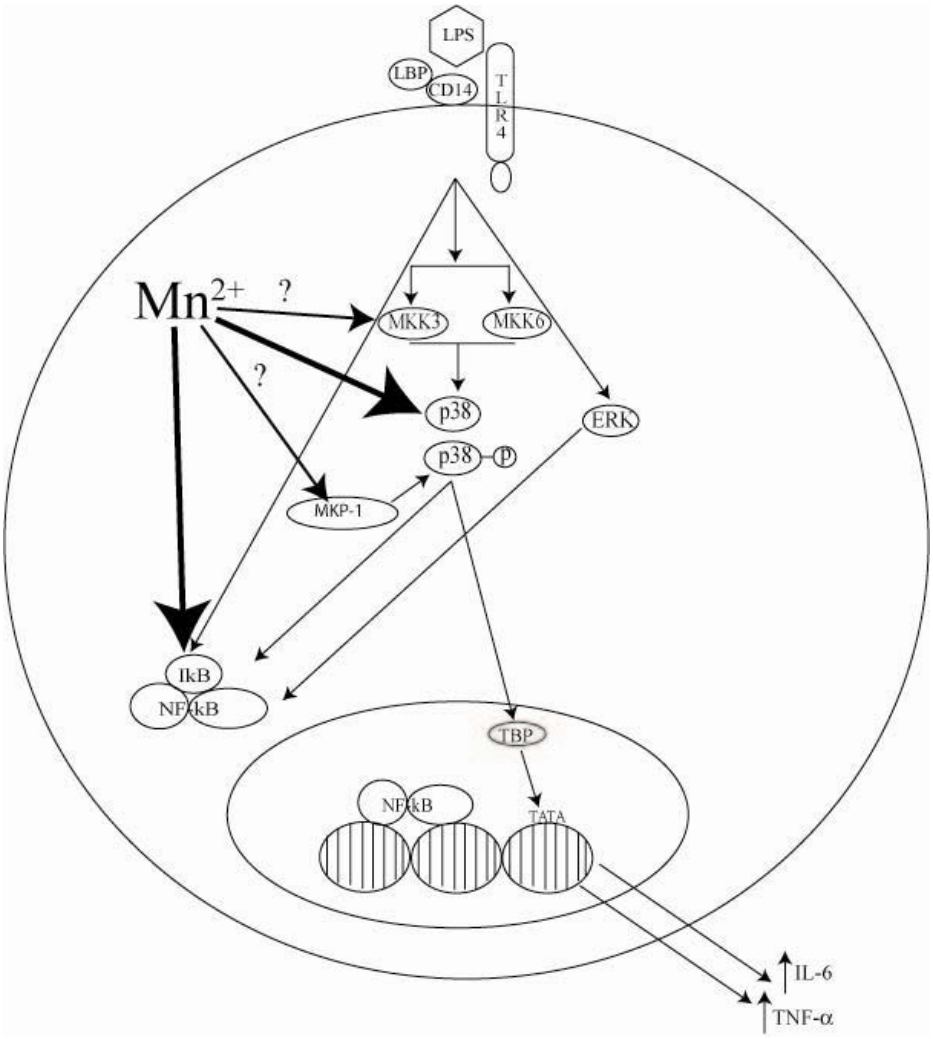

Figure 2.5 Diagram of potential/known Mn sites of action in microglial intracellular signaling following activation with LPS.

Small arrows indicate known intracellular signaling pathways while larger arrows indicate possible sites of interaction between signaling molecules and Mn. Large arrows with a '?' denote potential sites of action for Mn-enhanced p38 activity. Abbreviations: LPS (Lipopolysaccharide), LBP (LPS Binding Protein), TLR4 (Toll Like Receptor 4), MKK3/6 (Mitogen Activated Protein Kinase Kinase) p38 (p38 Mitogen Activated Protein Kinase), p38-p (phosphorylated p38), ERK (Extracellular Signal-Regulated Kinase), MKP-1 (Mitogen-activated protein kinase phosphatase-1), IkB (Inhibitor of NF-kB), NF-kB (Nuclear Factor kappa B), TBP (TATA-Binding Protein), TATA (TATA Box).

In summary, enhanced production of inflammatory cytokines by microglia exposed to Mn/LPS is associated with persistent activation of p38 and inhibition of p38 prevents this enhancement. Importantly, the increase cytokine production by Mn+LPS occurs regardless whether the exposure to Mn and LPS is concurrent or sequential (3 h apart); in all cases, the potentiation is prevented by inhibition of p38. Overall, our data suggest that exposure to environmental toxicants, such as Mn, may prolong microglial activation by utilizing the p38 pathway and contribute to chronic inflammation and neurotoxicity.

CHAPTER III

INCREASED P38 KINASE IN ACTIVATED MICROGLIA EXPOSED TO MANGANESE IS ASSOCIATED WITH DECREASED EXPRESSION OF THE PHOSPHATASE MKP-1 AND INCREASED PHOSPHORYLATION OF THE MKK-1 AND -4 KINASES

Abstract

In previous work we have demonstrated that manganese (Mn) exposure potentiates inflammogen (lipopolysaccharide; LPS)-induced inflammatory cytokine (e.g. IL-6 and TNF-α) production by microglial cells and that this potentiation is associated with enhanced and sustained p38 mitogen activated protein kinase (p38) activity. To explain the enhanced p38 activity, we hypothesized that Mn either augmented the activity of the kinases upstream of p38 (i.e. MKK-3/6, MKK-1/2, and/or MKK-4) or decreased activity of the major phosphatase responsible for p38 inactivation, MKP-1 (mitogen activated protein kinase phosphatase-1). We examined MKK activity following 1 or 4 h exposure to Mn (250 μM), LPS (100 ng/ml), or Mn+LPS and observed that Mn in combination with LPS increased phosphorylation of all three MKKs at 1 h, and MKK-3/6 at 4 h. However, for MKK-3/6, the observed activation by Mn+LPS was not different from the phosphorylation caused by LPS alone; indicating that the enhanced p38 activity

in the presence of Mn was not due to increased MKK-3/6 activation. In contrast to the lack of effect of Mn on MKK-3/6, Mn+LPS exposure increased the phosphorylation of MKK-1/2 and -4 at 1 h more than exposure to LPS alone, suggesting that effects of Mn+LPS on MKK-1/2 and -4 may lead to the previously observed enhanced p38 activity. Next, we examined the negative regulator of p38, MKP-1, by western blot and quantitative PCR. Exposure to Mn alone or in combination with LPS (1 h) decreased both mRNA levels and the protein expression of MKP-1 relative to the MKP-1 levels in control and LPS-exposed cells, respectively. Additionally, to correlate the sustained increase of p38 by Mn+LPS with the increased inflammatory mediator production (i.e. cytokines and prostaglandins), we performed a time-course mRNA study. Compared to the effects of LPS alone, further increase in TNF-α mRNA expression by Mn+LPS was observed as early as 1 h, while Mn+LPS markedly increased TNF-α, IL-6, and Cox-2 mRNA at 4 h, a time point coinciding with increased p38 activity. Thus, it appears that the enhanced and sustained p38 activity and, at least in part, inflammatory mediator expression by activated microglia exposed to Mn, may be due to decreased MKP-1 as well as enhanced MKK-1/2 and -4 activities.

Introduction

Manganese (Mn) is an essential metal and a common environmental contaminant. Besides mining operations, Mn is found in alloys, fertilizers, batteries, certain fungicides, and as a component of the fuel additive methylcyclopentdienyl manganese tricarbonyl (MMT; Frumkin and Solomon, 1997; Aschner, 2000). Excessive exposure to Mn is of human health concern, since occupational exposure to Mn is linked to a specific

neuropathology, manganism, that is characterized by clinical signs and lesions similar to Parkinson's Disease (PD; Calne *et al.*, 1994; Meco *et al.*, 1994; Frumkin and Solomon, 1997; Aschner, 2000; Huang *et al.*, 2007). Although Mn is thought to exert its effects, at least partially, by disrupting neuronal mitochondrial respiration, leading to increased oxidative stress and cell death (Gavin *et al.*, 1999), it has also been shown that Mn can potentiate the production of inflammatory mediators by non-neuronal cells resident in the CNS (Spranger *et al.*, 1998; Chang and Liu, 1999; Barhoumi *et al.*, 2004; Filipov *et al.*, 2005; Chen *et al.*, 2006; Crittenden and Filipov, 2008).

Inflammatory responses are essential for the maintenance and defense of tissues. However, uncontrolled or chronic inflammation can be detrimental to tissue homeostasis, especially in sensitive tissues such as the nervous system (Perry *et al.*, 1998). In fact, abnormally high levels of inflammatory cytokines, e.g. TNF-α, have been implicated in the etiology of PD (Nagatsu *et al.*, 2000). For example, astrocytes accumulate Mn and may produce reactive oxygen species (ROS) and other substances that may be damaging to neurons (Aschner, 2000). Importantly, it has been demonstrated that microglia, the resident brain macrophage, and/or astrocytes may produce inflammatory mediators that could be involved in the mechanisms of Mn neurotoxicity, especially in cases where an additional inflammatory stimulus such as lipopolysaccharide (LPS) is present (Spranger *et al.*, 1998; Chang and Liu, 1999; Filipov *et al.*, 2005; Crittenden and Filipov, 2008). LPS is a known environmental contaminant (Niehaus and Lange, 2003) and model inflammogen due to its ability to stimulate inflammatory cells (microglia/macrophages) to produce cytokines, nitric oxide (NO), and ROS (Chao *et al.*, 1992; Jeohn *et al.*, 2002b; Liu *et al.*, 2002).

Binding of LPS to CD14 and TLR4 cell surface receptors leads to the activation of intracellular kinases, including the mitogen activated protein kinases (MAPK; Bhat *et al.*, 1998; Jeohn *et al.*, 2002b). The MAPK family of proteins is comprised of a series of kinases, beginning with the MAP kinase kinase kinases (i.e. TAK1) that phosphorylate MAP kinase kinase (i.e. MKK-1, -2, -3, -4, -6), which subsequently phosphorylate MAPK. This MAPK cascade culminates in the activation of one or more MAPK, including the extracellular signal-regulated kinases (ERK), stress-activated or c-Jun N-terminal kinases (SAPK/JNK), and the p38 MAPK (Koistinaho and Koistinaho, 2002). Of these MAPKs, p38 MAPK (p38) and ERK appear to be primarily involved in the production of inflammatory mediators by microglia (Koistinaho and Koistinaho, 2002). MAPK deactivation is dependent on the actions of dual specificity phosphatases (DUSP), primarily MAP kinase phosphatase-1 (MKP-1; Koistinaho and Koistinaho, 2002; Lang *et al.*, 2006; Wang and Liu, 2007).

In primary microglia and microglial cell lines, LPS has been shown to increase the phosphorylation of ERK and p38, as well as increase the expression of iNOS and TNF-α in a time- and dose-dependent manner (Lee *et al.*, 1993; Lee *et al.*, 1994; Bhat *et al.*, 1998). Of note, LPS-induced, p38-dependent, increases in NO and TNF-α by microglia have been shown to decrease neuronal survivability in neuronal-glial co-culture (Jeohn *et al.*, 2002a). The fact that this effect can be inhibited by pretreatment with inhibitors of p38 suggests that p38 plays a dominant role in the process (Bhat *et al.*, 1998; Jeohn *et al.*, 2002b).

Within the context of Mn neurotoxicity, Mn enhances the production of inflammatory mediators by microglia. Indeed, exposure to Mn potentiates LPS-induced

production of inflammatory cytokines (TNF-α & IL-6) and NO *in vitro* (Filipov *et al.*, 2005; Crittenden and Filipov, 2008). In addition, Mn also potentiates Cox-2 expression and ensuing prostaglandin production by inflammogen-activated mixed glial cells (Liao *et al.*, 2007). Potentiating effect of Mn on TNF-α and IL-6 involves the activation of NF-kB and p38, as inhibitors of NF-kB and p38 were able to prevent the potentiation observed in Mn+LPS exposed cells (Filipov *et al.*, 2005; Crittenden and Filipov, 2008). Furthermore, the p38-mediated effect is independent of the timing of exposure to Mn, as Mn exposure 3 h before or after LPS, potentiated inflammatory cytokine production (Crittenden and Filipov, 2008). Whether the potentiating effects of Mn on cytokine production (and other inflammatory molecules) is at the level of transcription, and the temporal relationship with p38, is unknown is present.

Additionally, p38-phosphorylation alone did not explain the enhanced production of TNF-α and IL-6 in the presence of Mn+LPS, as LPS by itself increased the phosphorylation of p38 at earlier time points (up to 1 h) to levels comparable to the effects of Mn+LPS (Crittenden and Filipov, 2008). However, Mn+LPS exposure not only increased p38 activity, but also prolonged it up to 4 h following the initial exposure, a time point at which the LPS-increased p38 activity had subsided (Crittenden and Filipov, 2008).

The mechanism of prolonged p38 activation in the presence of Mn is not understood. It may be that Mn enhances, or prolongs, the activation of the MKKs responsible for the activation of p38, primarily MKK-3/6 (Enslen *et al.*, 1998). However, MKK-1/2 (Meja *et al.*, 2000) and MKK-4 (Derijard *et al.*, 1995) may also activate p38. Alternatively, decreased expression of MKP-1, the DUSP responsible for

dephosphorylating p38, may lead to prolonged p38 activation (Hammer *et al.*, 2005; Lang *et al.*, 2006; Wang and Liu, 2007).

To test these hypotheses, the major objectives of this study were to examine the effects of Mn+LPS exposure on the MKKs responsible for activating p38 (e.g. MKK-3/6, MKK-1/2, MKK-4) and on the major phosphatase responsible for deactivating p38, MKP-1. We have previously reported that Mn potentiates TNF-α and IL-6 production by LPS-activated microglia at the protein level (Filipov *et al.*, 2005), and that this potentiation is prevented by inhibition of p38 (Crittenden and Filipov, 2008). However, nor we or others have determined whether the potentiative effect of Mn on cytokine production occurs at the level of transcription. Therefore, an additional goal was to study the time-course of the expression of certain inflammatory mediators (e.g. TNF-α, IL-6, and Cox-2) in Mn+LPS-exposed microglia at the mRNA level, thus allowing us to determine whether the potentiating effect of Mn is at the transcription level and whether enhanced mRNA for TNF-α, IL-6, and Cox-2 by Mn+LPS coincides temporally with the previously observed increased p38 activity.

Materials and Methods

Chemicals. Unless specified, all chemicals and reagents were purchased from Sigma-Aldrich (Sigma; St. Louis, MO) and $MnCl_2$ with purity >99% was used.

Cell Culture. The N9 mouse microglial cell line (Righi *et al.*, 1989) was a gift kindly provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy) and is similar to primary microglia and other cell lines, i.e. N13, in that it produces inflammatory

cytokines, such as IL-1β, IL-6, and TNF-α, as well as NO when activated by inflammogens such as LPS (Righi *et al.*, 1989; Heyen *et al.*, 2000). The cultures were maintained (5% CO_2, 95% air, at 37°C) in RPMI-1640 supplemented with 10% Hyclone FBS (low endotoxin, ≤ 25 EU/ml; Hyclone, Logan, UT), 0.075% sodium bicarbonate, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 25 μg/ml gentamycin, 100 U/ml penicillin G, and 100 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA). For real-time quantitative PCR (qPCR) and western blot protein analysis, cells were seeded at 2.5 x 10^6 cells/well (5 ml volume) in 6-well plates (Costar) and allowed to adhere overnight prior to exposure to Mn or LPS. Cells were incubated for up to 4 h in the presence of Mn (250 μM) and/or LPS (*Escherichia coli* 0111: B4; 100 ng/ml).

In previous studies (Filipov *et al.*, 2005), Mn concentrations of 50-500 μM and LPS amounts of 10-1000 ng/ml were used, and the potentiation of cytokine production was both Mn- and LPS-dependent. Similar concentrations of Mn and LPS were used in this study based on previous experiments that indicated no significant N9 cell death following exposure to Mn and/or LPS at these concentrations (Filipov *et al.*, 2005). These observations are in line with numerous other *in vitro* studies where levels of Mn (Mn^{2+}) range from 10 μM to 4 mM with the most typical exposure range being 100 to 500 μM (Malthankar *et al.*, 2004; Li *et al.*, 2005). Moreover, the above concentrations and the concentrations used in our study are representative of Mn levels found in brains of non-human primates following exposure to manganese dioxide for 3 months (ranging from 35 to 350 μM; Suzuki *et al.*, 1975) and are in line with basal ganglia Mn levels observed in the brains of PD patients (Yase, 1972), as well as in autopsied brains of

patients with cirrhosis (Yase, 1972). Of note, the concentration of LPS we have used in the present study (100 ng/ml) is relatively low and we have already reported that when greater amounts of LPS are present in the culture medium, less Mn is required for a potentiating effect on cytokine production to be observed (Filipov *et al.*, 2005).

Dexamethasone (Dex) was used as a positive control for MKP-1, based on published data showing induction of MKP-1 in the presence of Dex, as well as the enhancement of LPS-induced expression of MKP-1 following exposure to both Dex+LPS (Zhou *et al.*, 2007).

Immunoblot analysis of MKK and MKP-1. After incubation for 1 and 4 h, cells were removed from the culture wells via scraping and the cell suspensions were centrifuged (300g; 10 min; 4°C). Following centrifugation, the supernatants were discarded, cells were resuspended in 100µl of RIPA (modified radioimmuno-precipitation) lysis buffer (1x PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing PMSF (Sigma), protease and phosphatase inhibitors (Protease Inhibitor Cocktail, Sigma, and Halt Phosphatase Inhibitor Cocktail, Pierce [Rockford, IL], respectively), and held on ice for 30 min with occasional pipetting to disrupt cell membranes. Protein concentration in the cell lysates was determined using the Bradford method with reagents obtained from Bio-Rad (Hercules, CA) and with bovine serum albumin (BSA) as a standard. Aliquots of each sample were diluted in reducing sample buffer and heat denatured for 5 min at 95 ºC. For gel electrophoresis, Kaleidoscope Molecular Weight Standard (Bio-Rad; Hercules, CA) was included as a western blot transfer control, while Magic Mark (Invitrogen; Carlsbad, CA) was used for molecular

weight determination and transfer control on the x-ray film. Twenty micrograms of total protein (MKK-3/6), 30 μg for MKK-1/2 and MKK-4, or 50 μg (MKP-1), was loaded and separated on a 10% SDS-PAGE gel, transferred to a PVDF membrane, and the membranes were blocked in 5% milk (MKK-3/6 and MKP-1) or fish gelatin (MKK-1/2 and MKK-4) for 1 h at RT. The membranes were then incubated overnight at 4ºC with antibodies specific for the non-phosphorylated (1:1000) and phosphorylated (1:500) MKK-3/6 (Cell Signaling Technology, Beverly, MA), phosphorylated (1:1000) MKK-1/2 and MKK-4 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and (1:500) MKP-1 protein (Santa Cruz). Following a wash (3x), the membranes were probed with either goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000, Bio-Rad) for MKK-3/6 and MKP-1, or rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (1:30,000, Santa Cruz) for MKK-1/2 and MKK-4. Next, the blots were exposed to SuperSignal West Pico chemiluminescent substrate (Pierce) for up to 5 min and then enclosed in transparent covers prior to exposure to x-ray film. Band density was analyzed using the UN-SCAN-IT software package (Silk Scientific Inc., Orem, UH).

qPCR analysis of MKP-1, IL-6, TNF-α, and Cox-2. Total RNA was isolated, including the recommended DNase treatment step, using the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.). One μg of total RNA was used to synthesize the first strand cDNA. First strand cDNA was synthesized with the RT^2 PCR Array First Strand Kit (SuperArray Bioscience Corporation, Frederick, MD). Using 10 ng starting RNA per

sample, expression of pro-inflammatory cytokines (TNF-α and IL-6), Cox-2, and MKP-1 were determined by qPCR (iCycler iQ, Bio-Rad) using mouse-specific, certified primers (SuperArray, Frederick, MD). Reaction mixtures were assembled in optical 96 well plates using the RT-PCR SYBR Green Master Mix (SuperArray). Amplifications were performed in an iCycler iQ (Bio-Rad) programmed for an initial step of 30 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. To check the quality of the products, the melting curve program was run after the above cycling program. The expression of GAPDH, which was verified of not being affected by vehicle/Mn/LPS treatment, was used as a house-keeping gene for all samples. Data are presented as relative induction (fold changes) of MKP-1, inflammatory cytokine, or Cox-2 normalized to GAPDH. Data shown represent samples from four independent experiments, and measurements were performed in duplicate.

Statistical Analysis. Data were analyzed using appropriate analysis of variance (ANOVA). When statistical differences were detected ($P < 0.05$), treatment means were separated by the Fisher's LSD *post hoc* test. All data are presented as means $\pm$ S.E.M.

Results

Immunoblot analysis of MKK and MKP-1. To determine the source of the enhanced/prolonged p38 activation observed in the presence of Mn (Crittenden and Filipov, 2008), we examined the induction of the MKK upstream of p38 (i.e. MKK-1/2, -3/6, -4), as well as its main negative regulator, the phosphatase MKP-1. Following exposure to Mn (250 μM), with and without LPS (100 ng/ml) for up to 4 h, levels of non-

phosphorylated MKK-3/6 (all exposures) did not change in response to Mn and/or LPS exposure. However, induction of MKK-3/6 phosphorylation was observed at 1 h post exposure to Mn+LPS or LPS alone; this effect persisted through the 4 h time point (Figures 3.1A and 3.1B, respectively). On the other hand, Mn by itself did not increase the phosphorylation of MKK-3/6 at any time and the effect of Mn+LPS was not greater than the effect of LPS (Figures 3.1A and 3.1B).

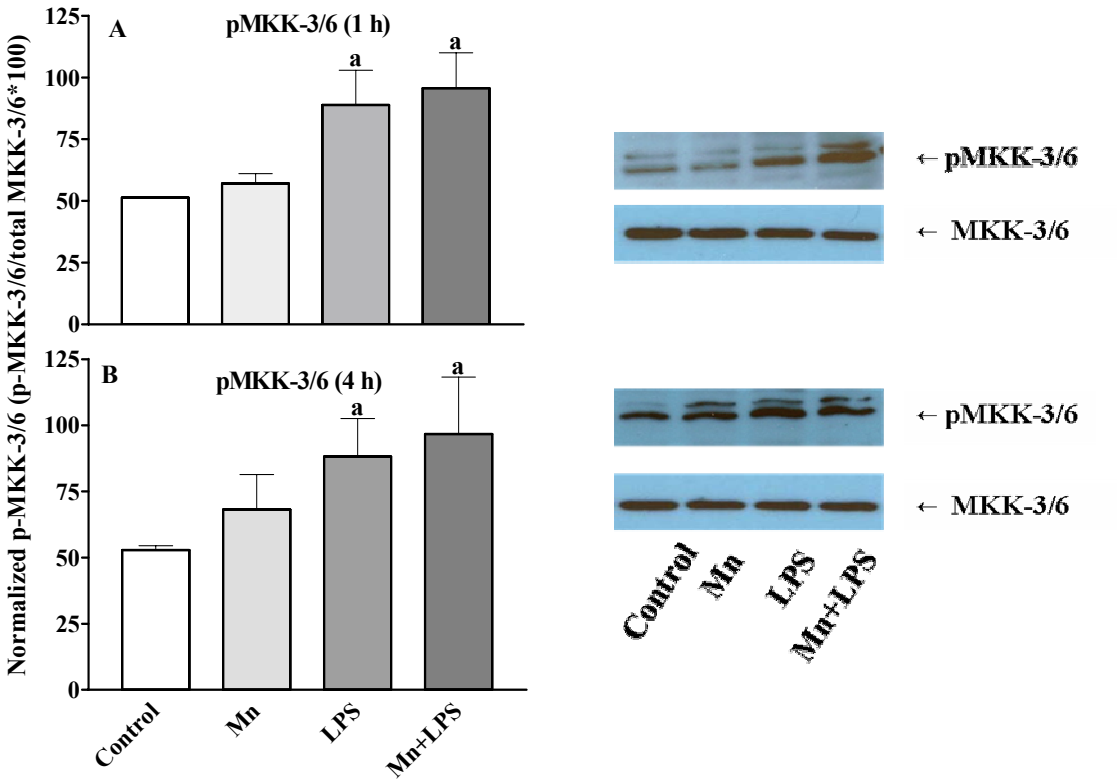

Figure 3.1 Quantification and western blots shown representative of MKK-3/6 phosphorylation following exposure to vehicle, 250 µM Mn, 100 ng/ml LPS, or 250 µM Mn + 100 ng/ml LPS for 1 (A) and 4 (B) h.

Densitometric data were adjusted relative to vehicle control and normalized as a ratio of phosphorylated to total (non-phosphorylated) MKK-3/6 protein. All data points represent means ± SEM from at least 3 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference at $p \leq 0.05$.

Similar to MKK-3/6, Mn alone did not cause appreciable phosphorylation of MKK-1/2 or MKK-4 at 1 or 4 h (Figures 3.2A, 3.2B, 3.3A, and 3.3B, respectively). However, at 1 h, the combination of Mn+LPS significantly increased the phosphorylation of both MKK-1/2 and MKK-4 to levels greater than the effect of LPS alone (Figures 3.2A and 3.3A). This effect was transient, as no statistical differences were observed in the presence of Mn or LPS for MKK-1/2 or MKK-4 at 4 h post exposure (Figures 3.2B and 3.3B).

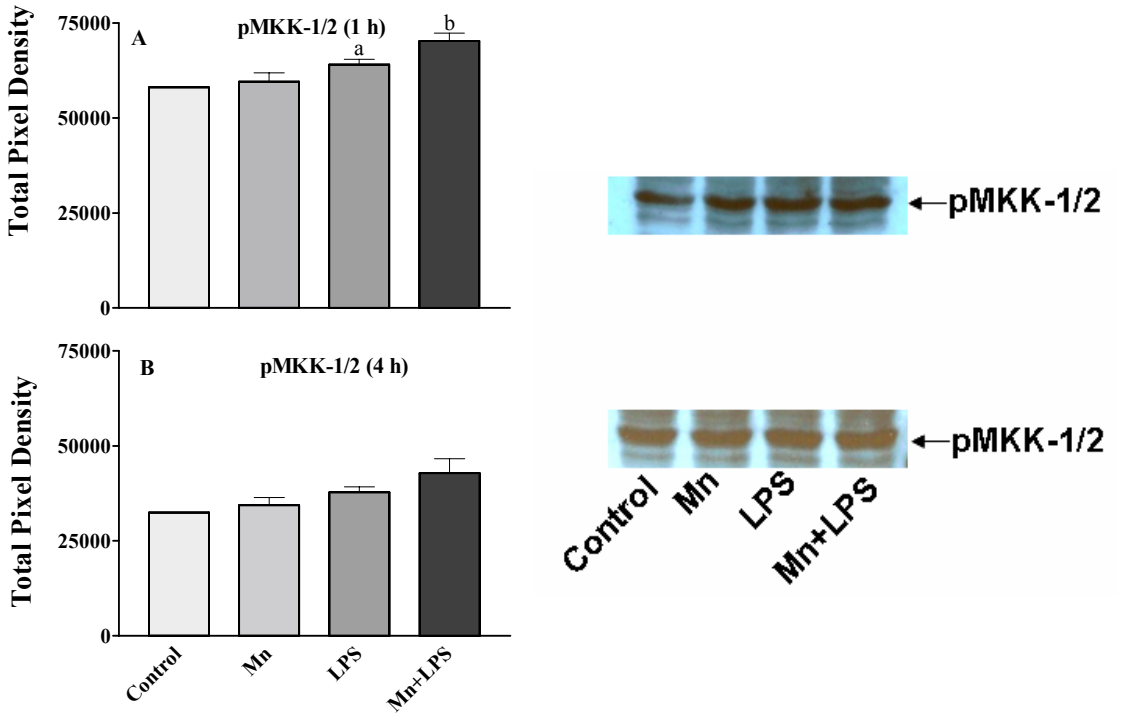

Figure 3.2 Quantification and western blots shown representative of MKK-1/2 phosphorylation following exposure to vehicle, 250 µM Mn, 100 ng/ml LPS, or 250 µM Mn + 100 ng/ml LPS for 1 (A) and 4 (B) h.

Densitometric data for phosphorylated MKK-1/2 are represented as total pixel density. All data points represent means ± SEM from at least 3 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference at $p \leq 0.05$.

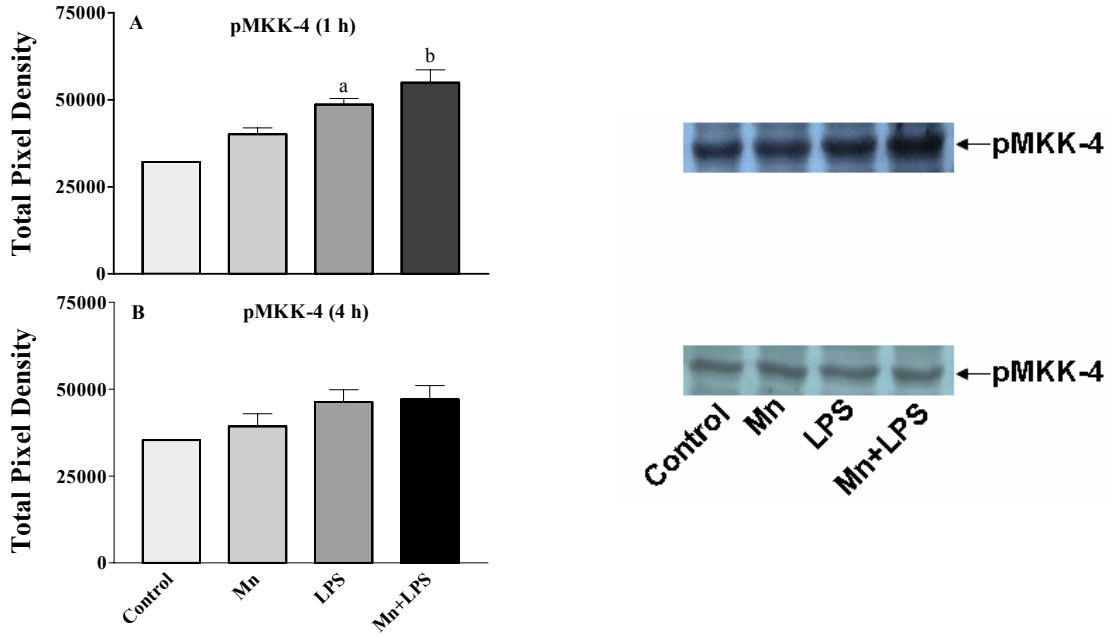

Figure 3.3 Quantification and western blots shown representative of MKK-4 phosphorylation following exposure to vehicle, 250 µM Mn, 100 ng/ml LPS, or 250 µM Mn + 100 ng/ml LPS for 1 (A) and 4 (B) h.

Densitometric data for phosphorylated MKK-4 are represented as total pixel density. All data points represent means ± SEM from at least 3 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference at $p \leq 0.05$.

Based on published observations in primary microglia (Zhou *et al.*, 2007), Dex with and without LPS was used as a positive MKP-1 control. Similar to primary microglia, Dex+LPS, but not Dex alone, was able to increase the MKP-1 protein levels at both 1 and 4 h in N9 microglia (Figures 3.4A and 3.4B). Dexamethasone alone decreased MKP-1 protein levels at 1 h, an effect that was not observed at 4 h (Figures 3.4A and 3.4B, respectively). In LPS- and Mn+LPS-treated cells, MKP-1 protein levels were also increased at 1 h (Figure 3.4A). However, MKP-1 protein levels in cells exposed to Mn were decreased relative to controls at the 1 h time point (Figure 3.4A). A similar decrease in MKP-1 levels was observed in Mn+LPS exposed cells relative to LPS alone (Figure 3.4A). Interestingly, the magnitude of the decrease was similar in both Mn and Mn+LPS exposed cells, relative to their respective controls (i.e. vehicle and LPS). At 4 h, a rebound effect was observed, as MKP-1 protein expression had significantly increased by in the Mn- and Mn+LPS-exposed cells (Figure 3.4B).

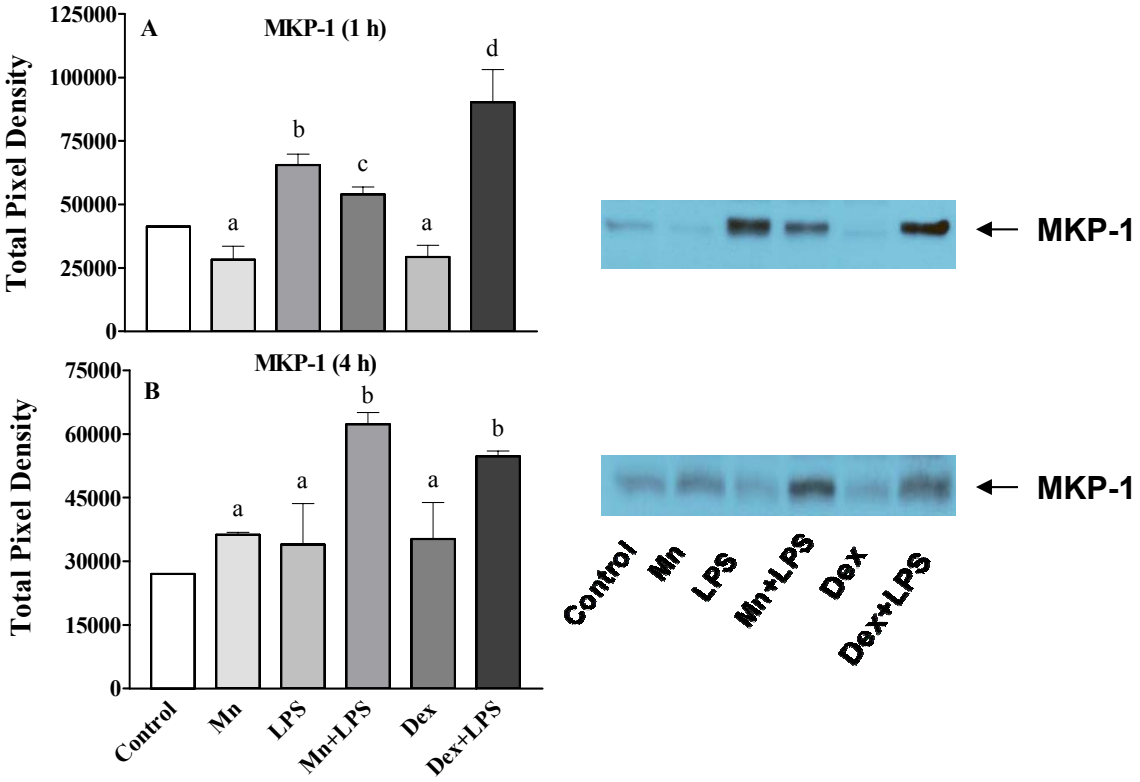

Figure 3.4 Quantification and western blots shown representative of MKP-1 following exposure to vehicle, 250 μM Mn, 100 ng/ml LPS, 250 μM Mn + 100 ng/ml LPS, Dex 1 μM, or Dex 1 μM + 100 ng/ml LPS for 1 h (A) and 4 h (B).

Densitometric data for MKP-1 are represented as total pixel density. All data points represent means ± SEM from at least 3 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference from control and bars with different letters are statistically different from each other at $p \leq 0.05$.

qPCR analysis of MKP-1. Similar to the MKP-1 protein levels, decreased expression of MKP-1 mRNA was observed for Mn-exposed cells at 1 h relative to the vehicle control (Figure 5A). Although Mn+LPS increased the overall expression of MKP-1 mRNA relative to the vehicle or Mn alone, the effect was less than that of LPS alone (Figure 3.5A). Interestingly, the magnitude of the change in MKP-1 mRNA expression in cells exposed to Mn-alone or in combination with LPS, relative to their respective controls (vehicle and LPS), was similar (almost 2-fold decrease; Figure 3.5). Exposure to Dex or LPS increased MKP-1 mRNA expression (2- and 5-fold, respectively). As expected, the largest increase in MKP-1 mRNA expression was observed in the presence of Dex+LPS, the positive control (7.5-fold; Figure 3.5A).

In contrast to the 1 h data, and in agreement with the western blot data, Mn increased the expression of MKP-1 mRNA at 4 h (Figure 3.5B). Although the amounts of MKP-1 mRNA in Dex, LPS, Mn+LPS, and Dex+LPS at 4 h were diminished in comparison to 1 h exposures, MKP-1 mRNA in Mn+LPS exposed cells was significantly increased in comparison to LPS alone at 4 h (Figure 3.5B).

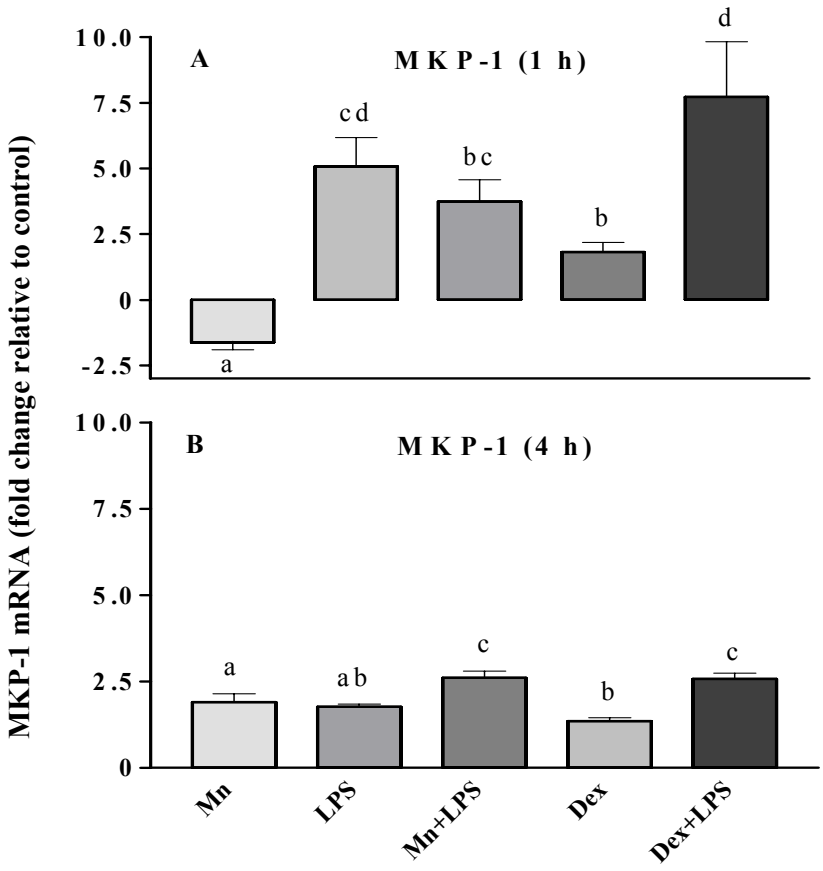

Figure 3.5 Fold change (up/down from vehicle control) in MKP-1 mRNA expression following exposure to 250 μM Mn, 100 ng/ml LPS, 250 μM Mn + 100 ng/ml LPS, Dex 1 μM, or Dex 1 μM + 100 ng/ml LPS for 1 (A) and 4 (B) h.

All data points represent means ± SEM from 4 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference from control and bars with different letters are statistically different from each other $p \leq 0.05$.

qPCR analysis of TNF-α, IL-6, and Cox-2. To examine the temporal expression of the inflammatory cytokines TNF-α and IL-6, as well as Cox-2, following exposure to Mn and/or LPS, we quantified TNF-α, IL-6, and Cox-2 mRNA at 1 and 4 h post exposure. Mn exposure by itself did not affect TNF-α mRNA at either time point (Figures 3.6A and 3.6B). Exposure to LPS, on the other hand, significantly increased TNF-α mRNA about 100-fold at 1 h in comparison to control, and this effect diminished by 4 h (Figures 3.6A and 3.6B). Similar to the effect observed with LPS alone, Mn+LPS significantly increased TNF-α mRNA at 1 h, with the effect of Mn+LPS on TNF-α expression being moderately greater than the effect of LPS alone (Figure 3.6A). While the LPS effect diminished substantially by 4 h, the Mn+LPS effect persisted, thus allowing for much greater TNF-α mRNA levels at 4 h in the Mn+LPS group compared to the LPS alone treatment (Figure 3.6B).

In accord with the TNF-α mRNA data, LPS significantly increased expression of IL-6 mRNA at 1 h and 4 h (Figures 3.6C and 3.6D). Similar to TNF-α, no effect on IL-6 mRNA expression was observed following exposure to Mn alone (Figures 3.6C and 3.6D). Interestingly, although Mn+LPS increased IL-6 expression at 1 h, in contrast to the effect of Mn+LPS on TNF-α, this effect was not greater than the effect of LPS alone (Figure 3.6C). However, similar to TNF-α, the expression of IL-6 mRNA in cells exposed to Mn+LPS increased over time from 1 to 4 h, almost 10-fold (Figure 3.6D). This effect was specific for Mn+LPS, as IL-6 mRNA in LPS-alone exposed cells,

although significantly greater than vehicle, did not increase between the 1 and 4 h time points (Figures 3.6C and 3.6D).

As recent data have suggested that non-cytokine inflammatory molecules, such as prostaglandin E-2 (PGE2), may be important in Mn-induced inflammation (Liao *et al.*, 2007), we also quantified Cox-2 mRNA in the presence of Mn and/or LPS at 1 and 4 h. Although exposure to LPS and Mn+LPS increased the expression of COX-2 mRNA by 20-fold at 1 h, the effect of Mn in combination with LPS was not greater than LPS alone (Figure 3.6E). However, similar to TNF-α and IL-6, by the 4 h time point, Mn+LPS exposure had increased Cox-2 mRNA even further to 30-fold. In contrast, the LPS-induced increase in Cox-2 mRNA, although still present, was only 11-fold (Figure 3.6F).

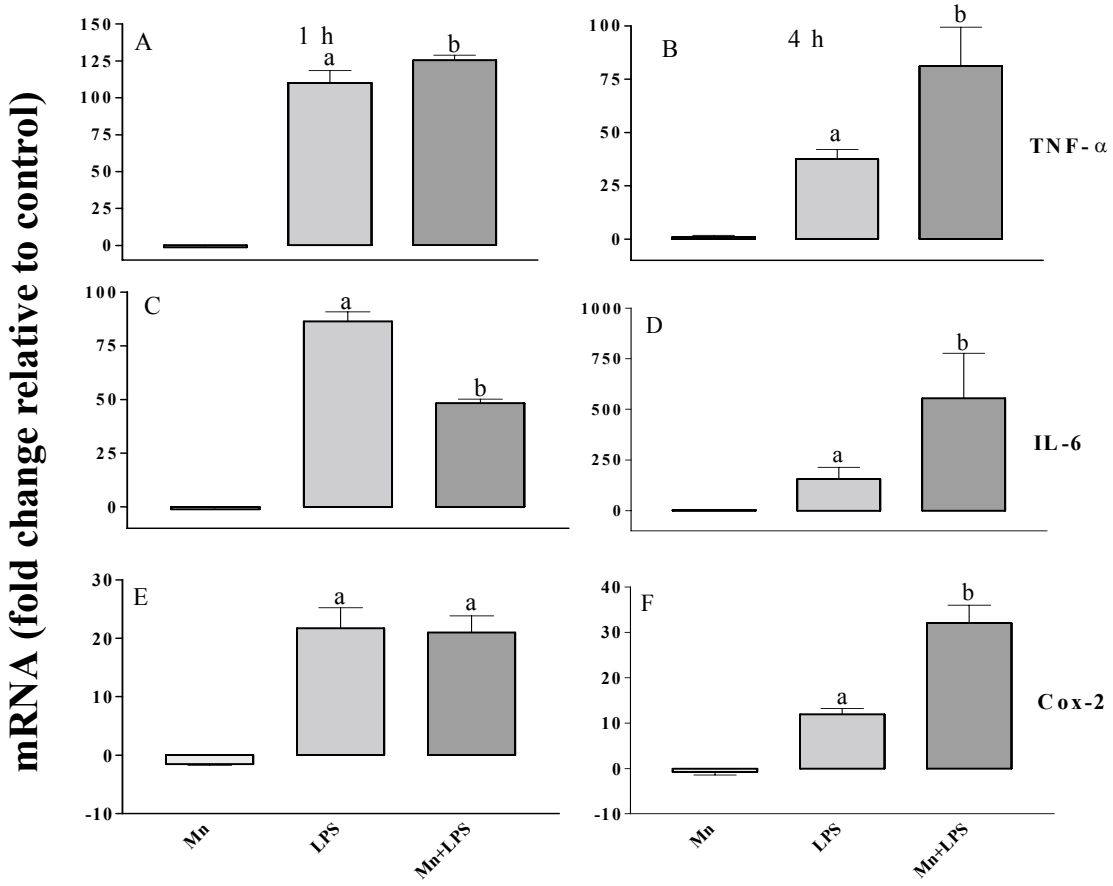

Figure 3.6 Fold change (up/down from vehicle control) in TNF-α (A and B), IL-6 (C and D), and Cox-2 (E and F) mRNA expression following exposure to 250 μM Mn, 100 ng/ml LPS, or 250 μM Mn + 100 ng/ml LPS for 1 h (left-hand graphs) and 4 h (right-hand graphs).

All data points represent means ± SEM from 4 independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. Letters denote statistically significant difference from control and bars with different letters are statistically different from each other $p \leq 0.05$.

Discussion

Previous research has demonstrated that Mn exposure, in combination with an inflammogen, can increase the production of iNOS/NO (Chang and Liu, 1999; Barhoumi *et al.*, 2004; Filipov *et al.*, 2005; Chen *et al.*, 2006), PGE2 (Liao *et al.*, 2007), as well of the inflammatory cytokines TNF- α (Filipov *et al.*, 2005; Chen *et al.*, 2006) and IL-6 (Filipov *et al.*, 2005) by glial cells. Although Mn alone increases cytokine production in microglia, the increase is small and only at high concentrations of Mn, suggesting that prior/ongoing microglial activation is necessary for Mn-potentiation of at least TNF-α and IL-6 to be observed (Filipov *et al.*, 2005). The cause of the Mn-induced potentiation is not understood. However, several potential mechanisms have been explored involving the TLR4 signaling pathway.

Activation of macrophages and microglia by LPS is thought to occur (primarily) by binding of LPS to TLR4, leading to activation of intracellular kinases (i.e. MAPK) and transcription factors such as NF-κB (Barton and Medzhitov, 2003; Takeda and Akira, 2004). Moreover, activation of the MAPK and NF-κB by LPS results in the production of inflammatory mediators such as NO and TNF-α (Lee *et al.*, 1993; Lee *et al.*, 1994; Bhat *et al.*, 1998). In earlier studies, the effects of Mn on the production of inflammatory molecules by activated glia was shown to involve the activation of the transcription factor NF-κB (Barhoumi *et al.*, 2004; Filipov *et al.*, 2005; Chen *et al.*, 2006). Additionally, this effect is associated with the increased activation of MAPK (Bae *et al.*, 2006), particularly p38 (Zhang *et al.*, 2007; Crittenden and Filipov, 2008), which appears to be long lasting (Crittenden and Filipov, 2008).

In our current study, we examined the key signaling molecules that may be responsible for the prolonged activation of p38. The upstream MAPKs, MKK-3 and -6, can activate p38, leading to the production of inflammatory mediators (Igarashi *et al.*, 2000; Fujishiro *et al.*, 2001). However, based on our results, the enhanced and prolonged p38 activation observed in Mn-exposed, LPS-stimulated microglia (Crittenden and Filipov, 2008) is not the result of enhanced MKK-3/6 activation. Indeed, at all time points examined in our study, the combination of Mn+LPS was no more effective than LPS alone in inducing MKK-3/6 phosphorylation.

Although MKK-3 and -6 are considered to be the kinases primarily responsible for phosphorylating p38, data also suggest that MKK-1, -4, and –7 may be involved in p38 phosphorylation (Jiang *et al.*, 1997; Chan-Hui and Weaver, 1998; Zarubin and Han, 2005). In the present study, Mn in combination with LPS increased phosphorylation of both MKK-1/2 and MKK-4 above the increase caused by LPS alone. This may contribute to the enhanced and prolonged p38 activation we have reported recently (Crittenden and Filipov, 2008). Of note, both kinases have been shown to activate other MAPKs, including ERK and JNK for MKK-1/2 and MKK-4, respectively (Koistinaho and Koistinaho, 2002). Considering that ERK did not appear to play as important of a role in the enhanced TNF-α and IL-6 production by Mn as did p38 (Crittenden and Filipov, 2008), increased MKK-1/2 activity by Mn+LPS may only play a partial role in the persistent activation of p38 associated with the enhanced cytokine production we observed in Mn+LPS exposed microglia (Crittenden and Filipov, 2008).

Another possible explanation for increased and/or prolonged p38 phosphorylation caused by Mn exposure which we investigated in this study is decreased activity of the

phosphatase MKP-1 which is responsible for deactivating p38. The DUSPs, particularly MKP-1, are important regulators of the innate immune responses. Overexpression of MKP-1 in macrophages has been shown to inhibit phosphorylation of MAPK and production of inflammatory cytokines (Chen *et al.*, 2002; Shepherd *et al.*, 2004; Zhao *et al.*, 2005). Conversely, reduced expression of MKP-1 results in increased production of the inflammatory cytokines TNF-α and IL-6 (Chi *et al.*, 2006; Hammer *et al.*, 2006). This observation is in line with our current findings demonstrating that exposure to Mn for 1 h leads to reduced expression of MKP-1 protein. We also observed that MKP-1 was inhibited by Mn, but Mn alone does not increase IL-6 and TNF-α (Filipov *et al.*, 2005). These data corroborate our previous findings where Mn alone increased p38 activity, but did not increase IL-6 and TNF-α cytokine production and further highlight the fact that persistent increase of p38 activity by Mn is necessary, but not sufficient, for the increased cytokine production previously observed (Crittenden and Filipov, 2008).

As TLR signaling not only promotes the expression of MKP-1 mRNA, but also prolongs the half-life of the transcript, it was necessary to examine both protein and mRNA expression in the presence of Mn (Hammer *et al.*, 2005). Similar to results we obtained for MKP-1 protein, we observed a decrease in MKP-1 mRNA expression following exposure to Mn for 1 h. Likewise, compared to the LPS alone treatment, the decreased MKP-1 mRNA level following exposure to Mn+LPS was of the same magnitude as the decrease caused by the Mn-only treatment (as compared to vehicle control). Thus, the reduction in MKP-1 mRNA and protein expression could result in prolonged p38 activation and enhanced pro-inflammatory cytokine production, as we have reported (Crittenden and Filipov, 2008).

We believe the reduction in MKP-1 mRNA and protein at 1 h leads to prolonged p38 activity, which in turn stimulates feedback inhibition of the p38 pathway by promoting MKP-1 expression. Indeed, activation of TLR4 by LPS has been shown to increase the expression of MKP-1 via MyD88, PKC, as well as other MAPK, including JNK and ERK, leading to feedback inhibition of p38 (Lang *et al.*, 2006). It is possible that the prolonged p38 activity observed in the presence of Mn, stimulates feedback inhibition that is not effective through the 1 h time point because of the reduced expression of MKP-1 caused by Mn. However, the 'rebound' of MKP-1 expression observed at 4 h in Mn-exposed cells indicates the cell is compensating, albeit too late, to prevent the prolonged activation of p38 and the enhanced production of inflammatory cytokines, such as TNF-α and IL-6 (Filipov *et al.*, 2005; Crittenden and Filipov, 2008) and, perhaps, other inflammatory mediators, such as PGE2 (Liao *et al.*, 2007).

Although we have previously reported increased production of inflammatory cytokines by microglia exposed to Mn+LPS for 24 h in vitro at the protein level (Filipov *et al.*, 2005; Crittenden and Filipov, 2008), we did not examine cytokine mRNA expression. Previous studies have suggested that Mn+LPS exposure results in increased TNF-α (Chen *et al.*, 2006) and Cox-2 (Liao *et al.*, 2007) mRNA expression by mixed glial cells at 6 h post exposure. In accord with these data, compared to the effects of LPS alone, we observed that TNF-α, IL-6, and Cox-2 mRNA levels in cells exposed to Mn+LPS were markedly greater at 4 h post exposure. In contrast, at 1 h post exposure, except for a moderate potentiating effect on TNF-α, presence of Mn in the culture medium did not enhance inflammatory mediator mRNA above the effects of LPS alone. On the contrary, LPS increased the expression of IL-6 mRNA more than Mn+LPS at the

1 h time point. However, by the 4 h time point, increased IL-6 mRNA expression persisted in Mn+LPS exposed cells, while the effect of LPS diminished. Intuitively, it would be reasonable to assume a correlation between an increased inflammatory mediator message expression and prolonged p38 activity. Indeed, as we reported (Crittenden and Filipov, 2008), Mn+LPS effects on p38 were not different from these of LPS alone at 1 h post exposure. However, by 4 h, LPS-caused activation of p38 was no longer present, while the presence of Mn in the exposure solution maintained p38 in its activated state.

Taken together, as depicted on Figure 3.7, early dysregulation of feedback control of MAPK signaling by Mn exposure, specifically MKK-1, MKK-4, p38, and MKP-1, is associated with enhanced inflammatory mediator mRNA expression by microglia and may lead to prolonged inflammation in brain tissue, resulting in excessive neuronal damage and death.

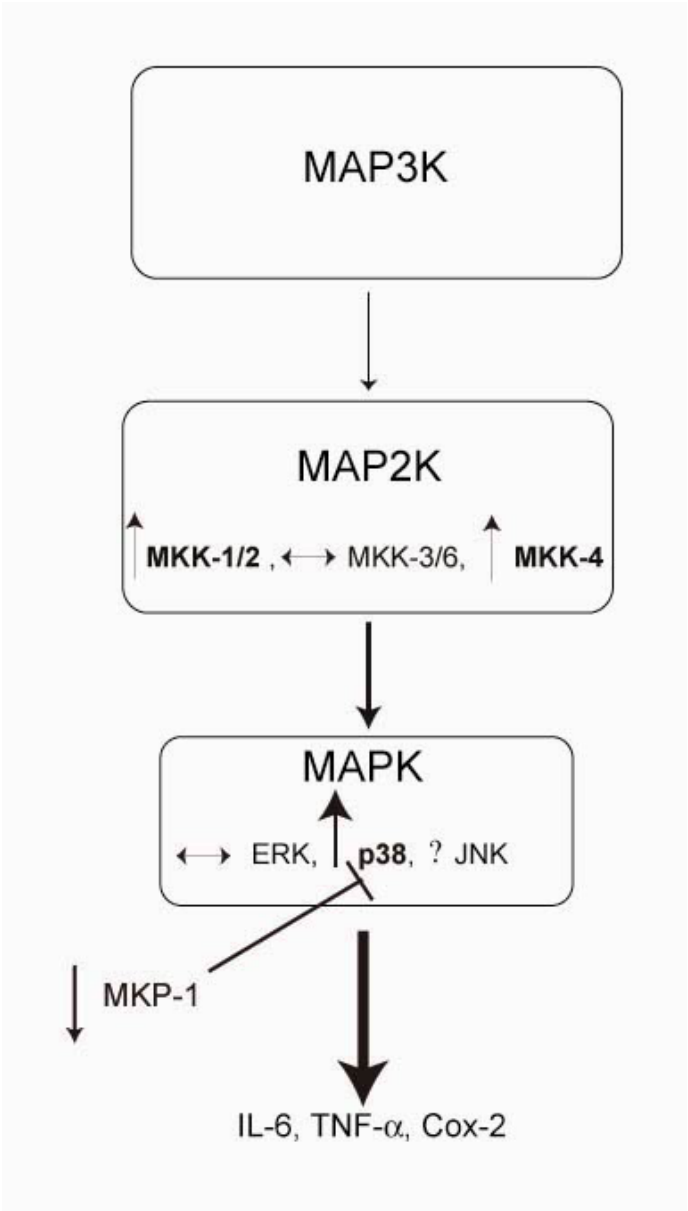

Figure 3.7 The effect of Mn on selected components of the MAPK cascade. Arrows indicate relative increase or decrease, with larger arrows indicating a greater effect than smaller arrows.

Abbreviations: MKK-1/2 (Mitogen Activated Protein Kinase Kinase-1/2), MKK-3/6 (Mitogen Activated Protein Kinase Kinase-3/6), MKK-4 (Mitogen Activated Protein Kinase Kinase-4), ERK (Extracellular Signal-Regulated Kinase), p38 (p38 mitogen activated protein kinase), JNK (c-Jun N-terminal Kinase), MKP-1 (mitogen activated protein kinase phosphatase-1).

CHAPTER IV

MANGANESE POTENTIATION OF MICROGLIAL INFLAMMATORY CYTOKINE PRODUCTION IS NOT RESTRICTED TO ACTIVATION VIA TLR4

Abstract

Previous studies have investigated the potentiation of lipopolysaccharide (LPS, a Toll-like receptor [TLR] agonist)-induced inflammatory mediators, such as NO and cytokines (IL-6 and TNF-α) by manganese (Mn) in primary microglia, microglial cell lines, and mixed glial cultures. Although the molecular mechanism(s) of the observed Mn-potentiation is not well understood, it is clear that transcription factors like NF-kB and prolonged activation of mitogen-activated protein kinases (MAPK), such as p38, are required. However, Mn-potentiation in microglial cells activated by TLR ligands other than LPS has not been explored. In the current study, to determine if Mn-potentiation was dependent on a specific TLR or TLR-associated signaling pathway, we examined Mn-potentiation of IL-6 and TNF-α in N9 microglia stimulated with ligands specific for TLR4 (LPS), TLR 2 (Deoxyribonucleic acid; CpG), TLR9 (Peptidoglycan; GPG), or TLR3 (Polyinosinic: polycytidylic acid; pI:C). In addition, we also examined the involvement of p38, ERK, and JNK in the potentiating effects of Mn. Manganese (24 h exposure) dose-respondantly potentiated secretion of pro-inflammatory cytokine proteins in all TLR-activated microglia. Additionally, Mn exposure potentiated TNF-α mRNA in

microglia activated with LPS, pI:C, CpG, or GPG at 4 h. The production of IL-6 mRNA at 4 h on the other hand, was differentially effected, with the greatest potentiating effect of Mn observed for LPS and pI:C. Although enhanced p38 activation by Mn was observed at 4 h following activation by all TLR-ligands, pharmacological inhibition of p38, JNK, or ERK decreased IL-6 and TNF-α production in all Mn-exposed, TLR-stimulated N9 microglia. These results indicate that Mn-potentiation is not limited to LPS-activated microglia, and potentiation can occur for an array of TLR-ligands working through at least four distinctive TLRs. Moreover, as Mn-potentiation occurred in both TLR2- and TLR3-stimulated cells, the observed potentiation may not be dependent on the TLR2/4/9-associated adaptor molecule, MyD88.

Introduction

Manganese (Mn), a ubiquitously distributed environmental contaminant (Aschner, 2000), in cases of excessive exposure can induce neurotoxicity that is characterized by a neurodegeneration that results in psychiatric symptoms and extrapyramidal neurologic deficits (Dobson *et al.*, 2004). Although the mechanism of this neurodegeneration is largely attributed to the ability of Mn to stimulate the generation of cytotoxic free radicals (Aschner and Aschner, 1991; Gavin *et al.*, 1999; HaMai and Bondy, 2004), it has been shown that neurodegeneration may also be due to the effects of Mn on the non-neuronal cells of the brain, astroglia and microglia. This is relevant in the context of Mn-induced inflammation, as it has been shown that glia, especially activated glia, produce significantly more NO, TNF-α, and IL-6 when exposed to Mn (Chang and Liu, 1999; Filipov *et al.*, 2005; Chen *et al.*, 2006; Crittenden and Filipov, 2008). Importantly,

increased expression of inflammatory mediators has been linked with neuronal cell injury and death (Liu *et al.*, 2002; Hald and Lotharius, 2005) and with elevated inflammatory cytokine expression in animal models of Parkinson's Disease (PD), as well as in the brains of PD patients (Nagatsu *et al.*, 2000; Sriram *et al.*, 2002). This is further supported by the observations that blockage or deletion of inflammatory mediators can attenuate basal ganglia neurotoxicity induced by the PD model toxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sriram *et al.*, 2002; Heales *et al.*, 1999; Ferger *et al.*, 2004).

Inflammation, while necessary for defense and tissue growth, may cause irreversible damage when prolonged or chronic in nature. In the brain, inflammatory mediators are produced by microglia and/or astroglia. Once activated by inflammogens such as lipopolysaccharide (LPS), a known environmental contaminant (Niehaus and Lange, 2003), these cells have been shown to produce inflammatory mediators like nitric oxide (NO), reactive oxygen species (ROS), and the cytokines interleukin-6 and tumor necrosis factor (IL-6 and TNF-α, respectively; Chao *et al.*, 1992; Jeohn *et al.*, 2002a; Jeohn *et al.*, 2002b; Liu *et al.*, 2002). Moreover, Mn has been shown to enhance the production of inflammatory mediators in LPS-activated glia (Filipov *et al.*, 2005; Liu *et al.*, 2005; Chen *et al.*, 2006; Crittenden and Filipov, 2008). Although the mechanism of Mn-potentiation of inflammatory mediator production in LPS-activated glia is still being explored, it has been demonstrated that several key intracellular signaling molecules are involved, all of which are activated by Toll-like Receptors (TLR; Filipov *et al.*, 2005; Liu *et al.*, 2005; Chen *et al.*, 2006; Bae *et al.*, 2006; Crittenden and Filipov, 2008).

Activation of cells of the innate immune system (i.e. macrophages and microglia) by LPS and other inflammogens (i.e. bacterial peptidoglycan, bacterial DNA, viral RNA), is thought to be primarily dependent on the activation of TLR (Beutler, 2004; Takeda and Akira, 2004). Perhaps the most well studied TLR activation pathway is the signaling initiated by binding of LPS to TLR4 (Beutler, 2004; Takeda and Akira, 2004). Binding of LPS to the TLR4 cell surface receptor initiates a signaling cascade via the adaptor molecule MyD88, which culminates in the activation of downstream kinases and transcription factors (Beutler, 2004; Takeda and Akira, 2004). In comparison, two other TLR that utilize the MyD88-dependent pathway for initiating intracellular activation are TLR2 and TLR9 when these receptors are 'activated' by their ligands, Gram positive bacterial cell wall peptidoglycan (GPG) and bacterial DNA (CpG), respectively (Beutler, 2004; Takeda and Akira, 2004). This is in contrast to MyD88-independent TLR-signaling that requires an alternative adaptor molecule, Toll-receptor-associated activator of interferon (TRIF), which is utilized primarily by TLR3 upon activation by its ligand, dsRNA (pI:C; Beutler, 2004; Takeda and Akira, 2004). Although primarily signaling through MyD88, evidence also suggests that, in contrast to TLR2 and TLR9, some of the TLR4 signaling may be MyD88-independent (Beutler, 2004; Takeda and Akira, 2004). Once activated, these TLR initiate a signaling cascade that culminates with the activation of NF-kB and the mitogen activated protein kinase (MAPK) cascade (Koistinaho and Koistinaho, 2002; Beutler, 2004; Takeda and Akira, 2004).

Activation of the MAPK cascade begins with the phosphorylation of the map kinase kinase kinase TAK1, which in turn phosphorylates 'downstream' map kinase kinases (MKK), MKK-1, -2, -3, -4, and -6, which subsequently phosphorylate their

'downstream' targets, the MAPK (Koistinaho and Koistinaho, 2002; Beutler, 2004; Takeda and Akira, 2004).

The MAPK are composed of three major kinases, including p38 MAPK (p38), extracellular signal-regulated kinase (ERK), and the stress-activated or c-Jun N-terminal kinase (JNK; Koistinaho and Koistinaho, 2002). Indeed, this is the case for activated primary microglia and microglial cell lines, as LPS stimulation increases the phosphorylation of p38, ERK, and JNK, as well as the expression of inflammatory mediators (Lee *et al.*, 1993; Lee *et al.*, 1994; Bhat *et al.*, 1998; Chen *et al.*, 2006). Furthermore, neuronal survivability in neuronal-glia co-culture is decreased in LPS-activated microglia, an effect that can be inhibited by pretreatment with inhibitors of p38 (Bhat *et al.*, 1998; Jeohn *et al.*, 2002a; Jeohn *et al.*, 2002b).

In the context of Mn-potentiation of inflammatory cytokines by LPS-stimulated microglia, we have demonstrated that potentiation is dependent on NF-kB and p38, as inhibitors of NF-kB and p38 were able to prevent enhanced IL-6 and TNF-α production in Mn+LPS cells (Filipov *et al.*, 2005; Crittenden and Filipov, 2008). Additionally, we have shown that p38 phosphorylation (activation) and p38 kinase activity is prolonged in the presence of Mn+LPS, relative to LPS alone, suggesting that the increased inflammatory cytokines in Mn+LPS-exposed cells is partly due to dysregulation of the p38 signaling pathway (Crittenden and Filipov, 2008). Moreover, we have demonstrated that this effect is independent of the timing of exposure to Mn, as exposure to Mn 3 h before or after LPS potentiated IL-6 and TNF-α production (Crittenden and Filipov, 2008). Furthermore, we have demonstrated that enhanced p38 activation is, in part, due

to enhanced activation of 'upstream' MKK-1/2 and -4, as well as decreased activity of the phosphatase, mitogen kinase phosphatase-1 (MKP-1; Chapter III).

However, the potentiating effect of Mn in microglia activated by TLR ligands other than LPS has not been studied and, perhaps, examining the distinctive TLR signaling pathways may provide information regarding the mechanism of Mn-potentiated inflammation previously observed for LPS-stimulated cells. To address these questions, in this study, our objectives were to (i) determine if Mn can potentiate inflammatory cytokine production (protein and mRNA) in N9 microglia stimulated with TLR-ligands other than LPS, (ii) if Mn-potentiation is observed for TLR-ligands other than LPS, determine whether p38 activation is associated with the Mn-potentiation, and (iii) using pharmacological inhibition, determine the role of the three major MAPK (i.e. p38, ERK, and JNK) for each Mn-TLR ligand combination.

Materials and Methods

Chemicals. Manganese ($MnCl_2$; 1 M stock) was purchased from Sigma-Aldrich (Sigma; St. Louis, MO) with purity >99.9 %, and was used for all experiments. TLR ligands utilized in this study were lipopolysaccharide (LPS, *Escherichia coli* 011:B4; Sigma), Polyinosinic-polycytidylic acid potassium salt (pI:C; Sigma), Peptidoglycan (GPG; Sigma), or CPG deoxyribonucleic acid (CpG; Cell Signaling, Inc.).

Cell Culture. For all experiments, the retroviral-immortalized brain microglial cell line, N9, was used. The N9 cell line was the kind gift of Dr. P. Ricciardi-Castagnoli (University of Milan, Italy), and is derived from day13 embryonic mouse brain cultures.

The N9 cell line is similar to another cell line, N13, as well as primary microglia, in that both produce inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, as well as NO when activated by LPS (Righi *et al.*, 1989; Heyen *et al.*, 2000).

All cultures were maintained at 37° C (5% CO2 with 95% air) in RPMI-1640 supplemented with 10% FBS (low endotoxin, ≤ 25 EU/ml; Hyclone, Logan, UT), 0.075% sodium bicarbonate, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM L-glutamax, 50 μM 2-mercaptoethanol, 25 μg/ml gentamycin, 100 U/ml penicillin G, and 100 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA). For pharmacological inhibition and cytokine studies, cells were seeded at 0.25 x 10^6 cells/well (0.5 ml volume) in 48-well plates (Costar; Fisher Scientific, Pittsburgh, PA). For western blot and mRNA analysis, cells were seeded at 2.5 x 10^6 cells/well (5 ml volume) in 6-well plates (Costar). Cells were incubated for up to 24 h in the presence of Mn (100 or 250 μM), Gram negative bacterial Lipopolysaccharide (LPS, *Escherichia coli* 011:B4; 100 ng/ml), synthetic viral RNA Polyinosinic-polycytidylic acid potassium salt (pI:C; 50 μg/ml), Gram positive bacterial peptidoglycan (GPG, *Staphylococcus aureus*; 5 μg/ml), or synthetic bacterial deoxyribonucleic acid (CpG; 200 nM).

The Mn and LPS exposures used in this study were consistent with reported environmental exposures and previous studies as described in previously published work (Crittenden and Filipov, 2008).

Cytokine Analysis. To quantify the amounts of IL-6 and TNF-α, we used DuoSet ELISA kits from R&D Systems, Inc, Minneapolis, MN) and manufacturer's instructions were followed. Briefly, 96-well microplates (Costar) were coated for

overnight at 4 C with their respective capture antibody in PBS. The plates were then washed with PBS-Tween buffer (3x) and blocked (1% BSA in PBS) for 1 h at RT. Then, the samples or standards were applied to individual wells and incubated for 2 h at RT. Next, the plates were washed 3x, incubated with detection antibody for 2 h, followed by another 3x wash. Strepavidin-HRP conjugate was added to each well for 20 min at RT, plates were washed 3x, and TMB-based substrate was added to each well and allowed to develop up to 30 min. To stop the reaction, 50 μl of 2N H_2SO_4 was added to each well and the absorbance was read using a Spectramax Plate Reader (Molecular Devices, Sunnyvale, CA) at 450 nm. For each cytokine, samples were assayed in duplicate and standard curves run on each plate were utilized to estimate the mean for each sample.

Pharmacological manipulation. For pharmacological inhibition of p38, JNK, and ERK, the inhibitors SB203580, SP600125, and UO126, respectively, (Calbiochem, San Diego, CA), were added 1 h prior to exposure to Mn and/or TLR ligand. The inhibitors were dissolved in DMSO (25.4 mM) and stored and –80°C until diluted to a working concentration of 2.5 mM prior to use. For actual experiments, inhibitors were diluted in the culture media to the final concentration of 25 μM. Thus, exposure to DMSO was at 0.1% for vehicle and MAPK-inhibitor exposed cells and it had no effect on cellular function.

qPCR analysis of IL-6 and TNF-α. Gene expression for IL-6 and TNF-a was determined using qPCR. Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and all samples were treated with DNase to remove any contaminating genomic DNA. Total RNA was quantified using the NanoDrop

Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and 1 μg of total RNA was used to synthesize the first strand cDNA with the RT^2 PCR Array First Strand Kit (SuperArray Bioscience Corporation, Frederick, MD). Starting with 10 ng of RNA per sample, expression of the cytokines IL-6 and TNF-α was determined by qPCR (iCycler iQ, Bio-Rad) using mouse specific primers (SuperArray). Each reaction mixture was combined within an individual well of a 96-well plate using the RT-PCR SYBR Green Master Mix (SuperArray) and amplifications were performed using an iCycler iQ (Bio-Rad). Reactions were amplified using an initial step of 30 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. To determine the quality of the products, a melting curve program was ran after the cycling program. For determination of baseline message expression, the expression of the house-keeping gene GAPDH was verified as not being affected by vehicle/Mn/LPS treatment. Data are presented as relative induction (fold-change) of pro-inflammatory cytokine. Data shown are representative of up to four independent experiments and measurements were performed in duplicate.

Immunoblot Analysis of p38 MAPK Following incubation for 1 or 4 h, cells were removed by scraping, centrifuged at 300 xg for 10 min a 4 °C, resuspended in 100 μl of RIPA (modified radioimmunoprecipitation) lysis buffer (1x PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing PMSF (Sigma), protease and phosphatase inhibitors (Protease Inhibitor Cocktail, Sigma, and HALT Phosphatase Inhibitor Cocktail, Pierce, [Rockford, IL], respectively), and held on ice for 30 min with occasional pipetting to disrupt cell membranes. The cell lysate protein concentration was determined using

the Bradford method with reagents obtained from Bio-Rad and with bovine serum albumin (BSA) as a standard. Sample aliquots were denatured in reducing sample buffer by heating for 5 min at 95 °C. Samples were electrophoretically separated by loading 20 μg of total protein per well in a 10% SDS-PAGE gel, transferred to a PVDF membrane, and the membranes were blocked in 5% milk for at least 1 h at RT.

All membranes were incubated overnight at 4 °C with antibody specific for phosphorylated p38 MAPK (1:500) or non-phosphorylated p38 MAPK (1:3000) (Cell Signaling Technology, Beverly, MA). Following incubation, each membrane was rinsed (3x) and washed (4x) for 15 min per wash. Then, each membrane was probed with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000, Bio-Rad) for 1.5 h at RT. Next, the blots were exposed to SuperSignal West Pico chemiluminescent substrate (Pierce) for up to 5 min and then enclosed in transparent covers prior to exposure to x-ray film. Band density was analyzed using the UN-SCANIT software package (Silk Scientific Inc., Orem, UH).

Statistical Analysis. Data were analyzed using appropriate analysis of variance (ANOVA). When statistical differences were detected ($P < 0.05$), treatment means were separated using Fisher's LSD *post hoc* test. All data are presented as means ± S.E.M.

Results

IL-6 and TNF-α protein expression. To determine whether Mn can potentiate inflammatory cytokine production by microglia activated by different TLR ligands, similar to the effects of Mn on LPS-activated microglia as we have previously reported (Filipov *et al.*, 2005; Crittenden and Filipov, 2008), we exposed N9 microglia to LPS, pI:C, CpG, or GPG, alone or in combination with Mn (100 or 250 μM), for 24 h. As previously reported, except for a small increase TNF-α by Mn (250 μM; Figures 4.1A), neither vehicle nor Mn alone induced significant cytokine (Figures 4.1A and 4.1B; Filipov *et al.*, 2005; Crittenden and Filipov, 2008). Inflammatory cytokine (IL-6 and TNF-α) production was significantly increased in microglia exposed to LPS, pI:C , CpG, and GPG (Figures 4.1A and 4.1B). The increase in media TNF-α levels was highest for cells exposed to LPS and pI:C (Figure 4.1A). While not as effective as LPS or pI:C at the concentrations used, CpG and GPG also significantly increased microglial TNF-α production (Figure 4.1A). Similar results were observed for IL-6, as all four TLR ligands increased IL-6 protein in the supernatant, with the highest levels of cytokine observed in pI:C and LPS exposed cells (Figure 4.1B).

Co-exposure to Mn (100 or 250 μM) and LPS, pI:C, CpG, or GPG potentiated TNF-α and IL-6 in a dose-responsive manner (Figures 4.1A and 4.1B). The most profound effect on TNF-α potentiation was observed in cells co-exposed to 250 μM Mn and CpG or GPG with a 3-fold increase in TNF-α compared to TLR ligand alone, although significant potentiation of TNF-α by Mn was observed for LPS and pI:C as well (Figure 4.1A). Manganese potentiated IL-6 production for all TLR ligands (LPS, pI:C,

CpG, GPG), with the greatest effects observed for 250 μM Mn in combination with pI:C or CpG, with overall increases of 3- and 4-fold (respectively) in comparison to the TLR ligands alone (Figure 4.1B).

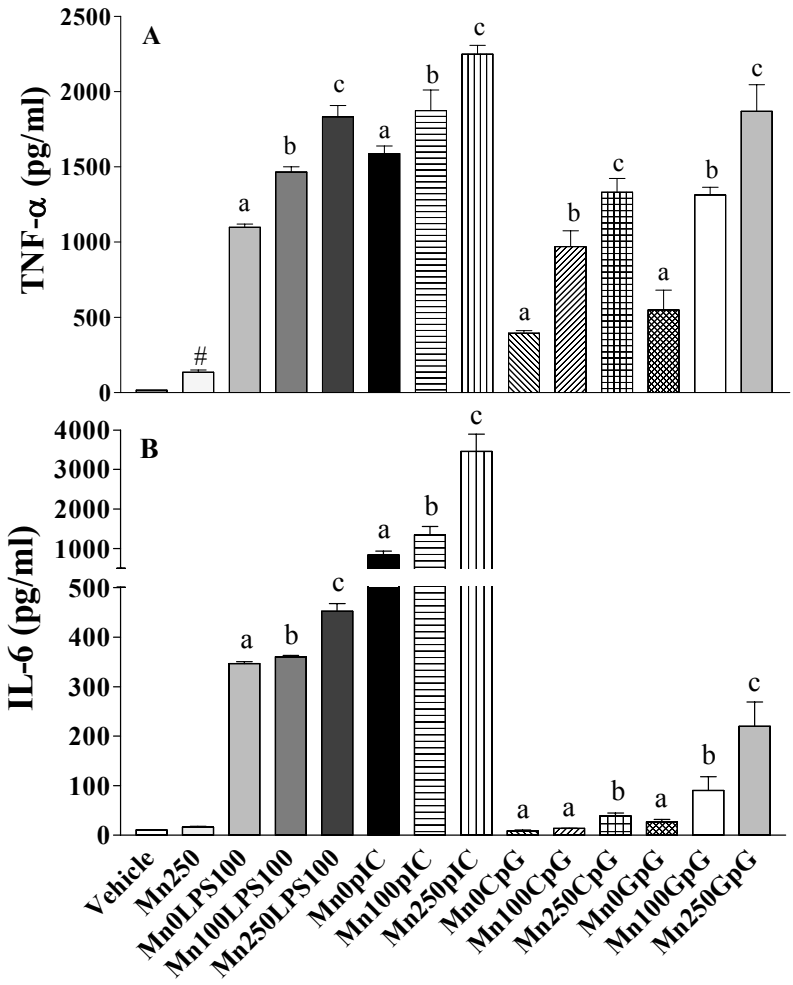

Figure 4-1 Effect of exposure to manganese (Mn) and/or lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (pI:C), peptidoglycan (GPG), microbial DNA (CpG) on microglial inflammatory cytokine production.

TNF-α (1A) and IL-6 (1B) production was analyzed in supernatants collected 24 h after exposure to N9 microglial cells to Mn (100 or 250 μM), and/or LPS (100 ng/ml), pI:C (50 μg/ml), CpG (200 nM), GPG (5 μg/ml). Media levels of TNF-α and IL-6 were analyzed by respective DuoSet ELISA as described in Materials and Methods. Data shown in each bar represent the mean ± SEM, of 4 independent replicates. [a,b,c] Presence of letters on top of bars indicate treatment differences relative to vehicle/Mn 250 or within a particular TLR ligand-Mn combination for TNF-α (1A) and IL-6 (1B) levels with bars with different letters being different from each other and from bars without a letter ($p \leq 0.05$). # Indicates significant effect of Mn ($p \leq 0.05$)

IL-6 and TNF-α mRNA expression. To determine whether Mn-potentiation of TNF-α and IL-6 occurred at the level of mRNA transcription, inflammatory cytokine mRNA expression was quantified using q-PCR. The TLR ligands alone significantly increased TNF-α and IL-6 mRNA expression, respectively (Figure 4.2A and 4.2B, respectively).

Manganese potentiated TNF-α mRNA expression for all TLRs at 4 h (Figure 4.2A). The highest TNF-α mRNA potentiation by Mn was observed for LPS and pI:C (2.5 and 4-fold increases, respectively), with a smaller, but significant, increase observed for CpG and GPG as well (Figure 4.2A). Somewhat in contrast to TNF-α mRNA induction, IL-6 mRNA expression was significantly potentiated by Mn in only the LPS- and pI:C-exposed cells, while the Mn-potentiation observed in CpG- or GPG-stimulated cells was only marginal and did not reach significance (Figure 4.2B).

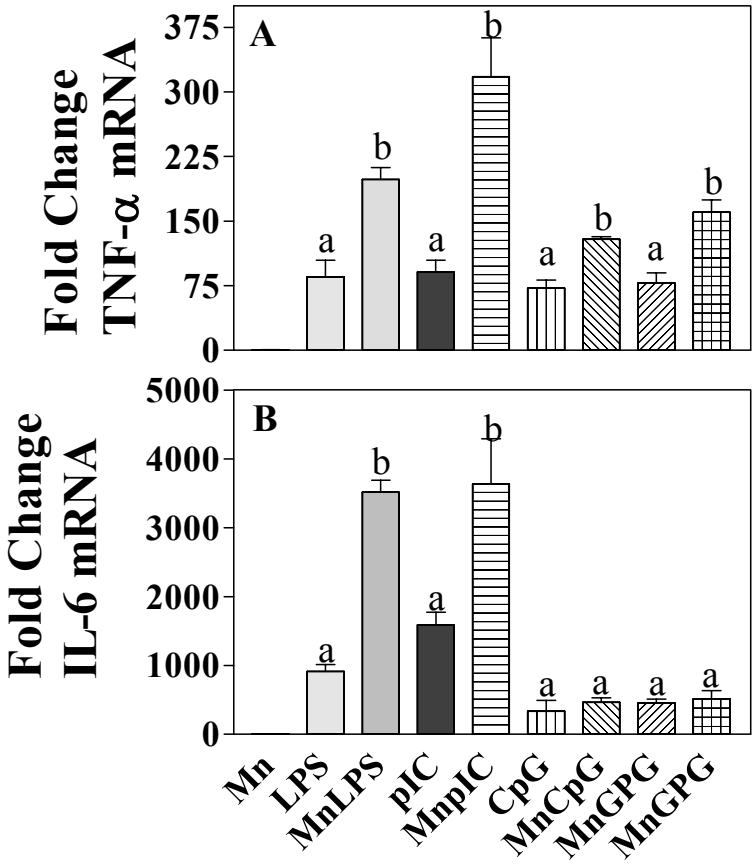

Figure 4.2 Fold change (up/down from vehicle control) in TNF-α (A) and IL-6 (B) mRNA expression following exposure to 250 μM Mn and/or LPS (100 ng/ml), pI:C (50 μg/ml), CpG (200 nM), GPG (5 μg/ml).

All data points represent means ± SEM from 3 or more independent experiments. Data were analyzed with ANOVA and means were separated using Fisher's LSD multiple comparison *post hoc* test. [a,b] Letters denote statistically significant difference at $p \leq 0.05$ and represent differences from vehicle and Mn within particular TLR ligand-Mn combination.

Immunoblot analysis of p38 and phospho-p38 protein. To examine p38 activation, we measured p38 phosphorylation (normalized to non-phosphorylated p38) following exposure to TLR-ligands alone, or in combination with 250 μM Mn at 4 h. Similar to previously published data (Crittenden and Filipov, 2008), LPS-induced p38 phosphorylation had subsided by 4 h and was not different from control, while 250 μM Mn- or 250 μM Mn+LPS-induced p38 phosphorylation was still significantly increased in comparison to vehicle control (Figure 4.3A). Similar to LPS alone at 4 h, pI:C, CpG, or GPG-induced p38 phosphorylation was not different from control (Figure 4.3A). However, significantly increased p38 phosphorylation was observed for 250 μM Mn in combination with pI:C, CpG, or GPG, with the largest increase being a 5-fold increase in p38 phosphorylation for CpG (Figure 4.3A). The levels of non-phosphorylated p38 (all exposures) did not significantly change in response to Mn and/or TLR ligands (Figure 4.3A).

In comparison to previous work examining the effect of 1 h exposure to Mn and LPS on p38 activation (Crittenden and Filipov, 2008), we quantified p38 phosphorylation in N9 microglia exposed to Mn and another TLR ligand, pI:C, for 1 h. Co-exposure to Mn and pI:C significantly increased p38 phosphorylation relative to pI:C alone, while the level of p38 phosphorylation in cells exposed to Mn or pI:C was greater than control but similar between the two (Figure 4.3B). Furthermore, in agreement with previously published data for Mn and LPS (Crittenden and Filipov, 2008), the p38 phosphorylation in microglia exposed only to pI:C diminished by 4 h (Figure 4.3A).

A

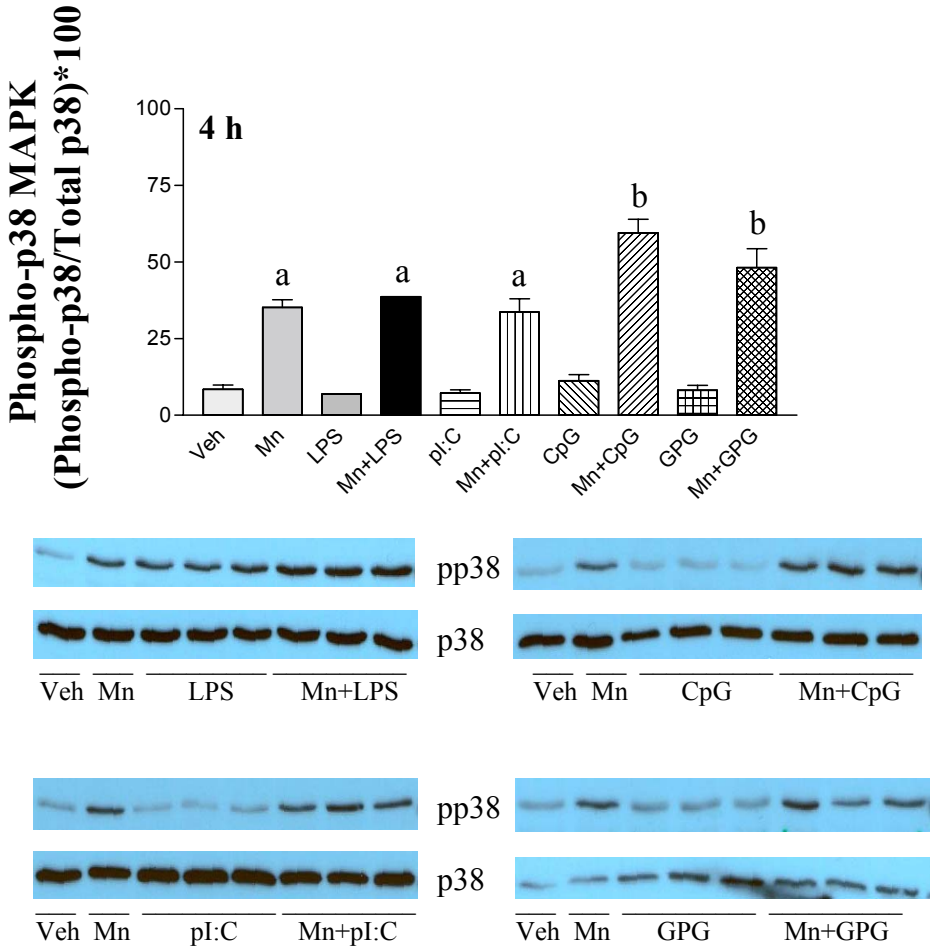

Figure 4-3 Analysis of phosphorylated p38 kinase in N9 microglia exposed to (A) 250 μM Mn and/or LPS (100 ng/ml), pI:C (50 μg/ml), CpG (200 nM), GPG (5 μg/ml) for 4 h or (B) 250 μM Mn and/or pI:C (50 μg/ml) for 1 h.

Western blots and densitometric analysis were performed as described in the Materials and Methods. Data shown represent the means ± SEM of the adjusted pixel density for phosphorylated p38 normalized to the non-phosphorylated p38. For abbreviations please refer to the legend on Fig.4-1. Each bar represents a minimum of 3 independent replicates. [a,b] Letters denote statistically significant difference at $p \leq 0.05$ and represent differences from vehicle, Mn, and/or TLR control.

B

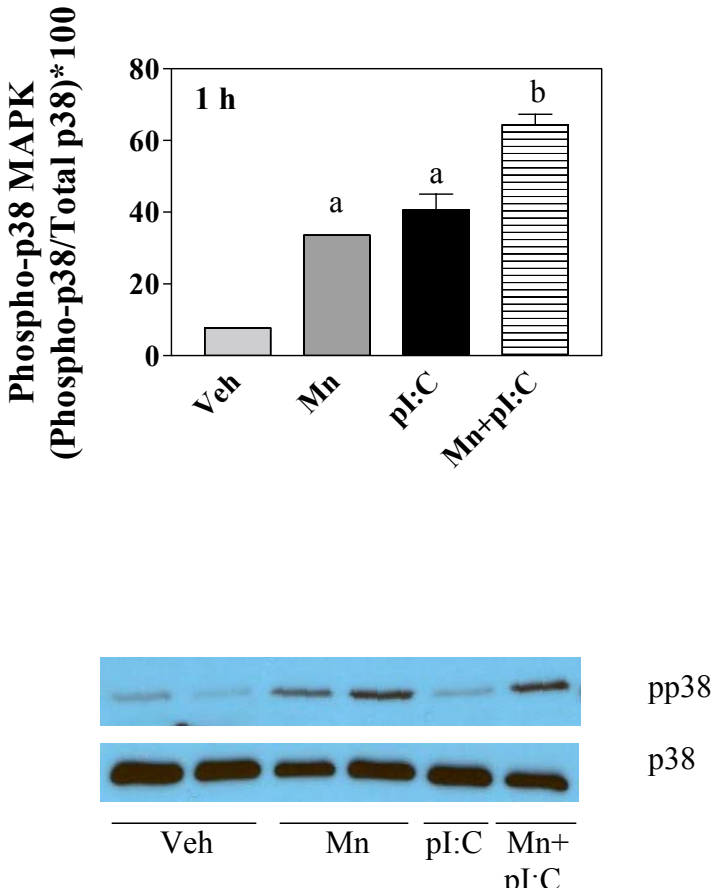

Figure 4-3 (continued)

Effect of MAPK inhibitors on cytokine expression. To determine whether p38, JNK, or ERK MAPK are required for Mn-potentiation of TNF-α or IL-6 in TLR-activated microglia, N9 cells were pretreated with inhibitors specific for either p38, JNK, or ERK. As previously observed, p38 inhibition prevented both LPS-induced TNF-α protein expression, as well as Mn-potentiation of LPS-induced TNF-α production (Figure 4.4A). Similarly, inhibition of JNK and ERK also prevented LPS-induced TNF-α production, as well as Mn+LPS-induced TNF-α production (Figure 4.4A).

As observed with LPS-stimulated microglia, production of TNF-α in microglia stimulated with CpG- or GPG-alone, or in the presence of Mn, was significantly inhibited by all three inhibitors, with the greatest effect observed for JNK- and ERK-inhibitors with near complete inhibition (Figures 4.4C and 4.4D). Unlike the previous TLR ligands (LPS, CpG, and GPG), inhibition of TNF-α production in pI:C- or Mn+pI:C-activated cells was greatest following inhibition of JNK or ERK, although all three inhibitors were effective (Figure 4C).

As we have also previously observed for IL-6, prior exposure to SB203580 significantly inhibited IL-6 production in LPS- and Mn+LPS-exposed cells (Figure 4.5A). However, SP600125 and UO126 were the most effective at inhibiting IL-6 production in LPS-stimulated cells (Figure 4.5A). Similar to the inhibition observed with LPS-stimulated cells, all MAPK inhibitors were effective at preventing IL-6 production in CpG- or GPG-stimulated N9 microglia, with the greatest effect observed for SP600125 and UO126 (Figures 4.5C and 4.5D). In comparison to TNF-α, prior exposure to

SB203580, SP60025, or UO126 prevented pI:C and Mn+pI:C-induced IL-6 production, with the greatest effect observed for the p38 and JNK inhibitors (Figure 4.5B).

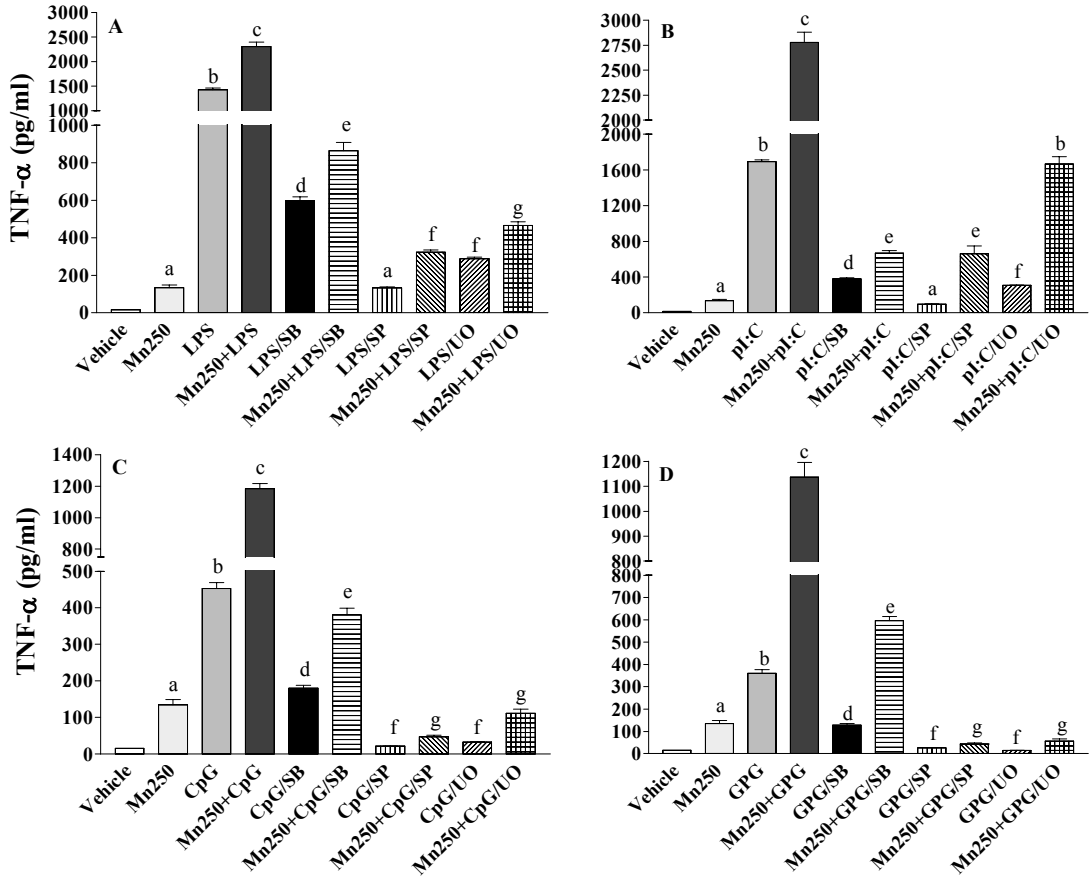

Figure 4.4 Effect of exposure to SB203580 (SB, p38 inhibitor), SP600125 (SP, JNK inhibitor), and UO126 (UO, ERK inhibitor) on Mn-potentiation of LPS, pI:C, CpG, or GPG-activated microglial TNF-α production.

TNF-α production was analyzed in N9 microglia exposed for 1 h to 25 μM of SB, JNK, or ERK, followed by exposure to 250 μM Mn and/or 100 ng/ml LPS (A), 50 μg/ml pI:C (B), 200 nM CpG (C), or 5 μg/ml GPG (D). Media levels of TNF-α and IL-6 were analyzed by respective DuoSet ELISA as described in Materials and Methods. Data shown in each bar represent the mean ± SEM, of 4 independent replicates. [a,b,c,d,e,f,g] Presence of letters on top of bars indicate treatment differences in the TNF-α levels with bars with different letters being different from each other and from bars without a letter ($p \leq 0.05$).

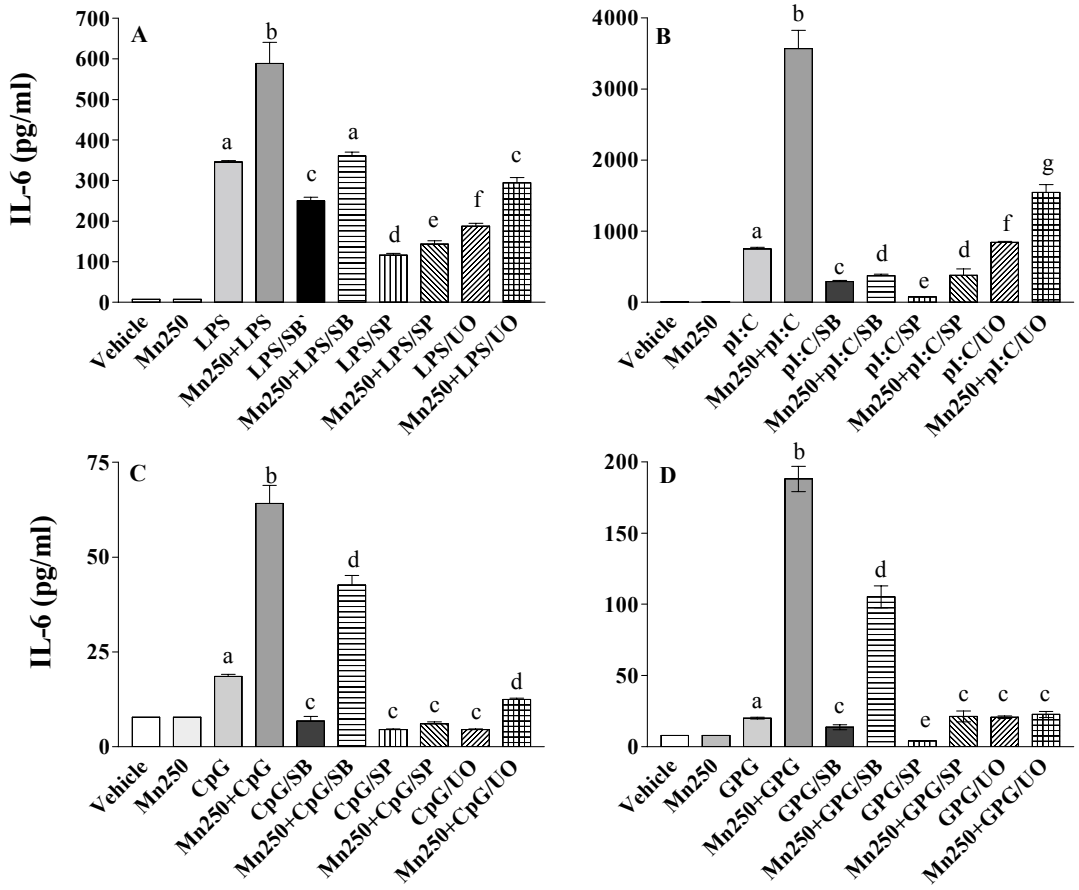

Figure 4.5 Effect of exposure to SB203580 (SB, p38 inhibitor), SP600125 (SP, JNK inhibitor), and UO126 (UO, ERK inhibitor) on Mn-potentiation of LPS, pI:C, CpG, or GPG-activated microglial IL-6 production.

IL-6 production was analyzed in N9 microglia exposed for 1 h to 25 μM of SB, JNK, or ERK, followed by exposure to 250 μM Mn and/or 100 ng/ml LPS (A), 50 μg/ml pI:C (B), 200 nM CpG (C), or 5 μg/ml GPG (D). Media levels of IL-6 were analyzed by respective DuoSet ELISA as described in the Materials and Methods. Data shown in each bar represent the mean ± SEM, of 4 independent replicates. . [a,b,c,d,e,f,g] Presence of letters on top of bars indicate treatment differences in the IL-6 levels with bars with different letters being different from each other and from bars without a letter ($p \leq 0.05$).

Discussion

Several previous studies have shown that Mn can enhance, or potentiate, LPS-induced inflammatory mediator production in glial cells (Chang and Liu, 1999; Filipov *et al.*, 2005; Chen *et al.*, 2006; Crittenden and Filipov, 2008). Additionally, it has been demonstrated that Mn-potentiation of neuroinflammation is dependent on NF-kB, and is associated with persistent p38 activation (Filipov *et al.*, 2005; Crittenden and Filipov, 2008). However, it is not clear if these effects are limited to a specific inflammagen (i.e. LPS) or activating receptor/pathway (i.e. TLR4). It has already been shown that primary microglia, both human and murine, produce TNF-α and IL-6 in response to stimulation with GPG, pI:C, LPS, and CpG which are ligands for TLR 2, TLR3,TLR4, and TLR9, respectively (Olson and Miller, 2004; Jack *et al.*, 2005; Town *et al.*, 2006). In the current study, following TLR stimulation, we observed similar increases in TNF-α and IL-6, as was observed for primary microglia (Olson and Miller, 2004; Jack *et al.*, 2005; Town *et al.*, 2006), suggesting that this (N9) cell line behaves similar to primary microglia. Moreover, the current results clearly demonstrate that not only does Mn-potentiation occur in TLR-stimulated N9 microglia for several TLR ligands, but that it does so in a dose-responsive manner (Figures 4.1 and 4.1B). Importantly, this suggests that Mn-potentiation is not dependent on MyD88, a common signaling adaptor for TLR2, TLR4, and TLR9, but not TLR3.

Of note, the increase in TNF-α and IL-6 due to the combination of Mn+TLR-ligand is not limited to protein expression, as increased mRNA was observed at 4 h for each cytokine (Figures 4.2A and 4.2B). Interestingly, although Mn-potentiated TNF-α mRNA for LPS, pI:C, CpG, and GPG-stimulated cells, IL-6 mRNA was only statistically

potentiated for LPS and pI:C (Figures 4.2A and 4.2B). This suggests that Mn-potentiation of CpG- and GPG-induced IL-6 production may not be entirely due to enhanced transcription of IL-6 mRNA. It should be noted that Mn alone did not increase cytokine protein or mRNA (Figures 4.1 and 4.2) to any major extent, suggesting that Mn-potentiation of TLR-induced cytokine mRNA and protein expression requires activation of a TLR-signaling pathway by a TLR agonist.

Previously, we have reported that Mn-potentiation of LPS-induced inflammatory cytokine production is associated with prolonged p38 activity (Crittenden and Filipov, 2008). In the present study, p38 phosphorylation was greatest in Mn+TLR-ligand exposed cells. Furthermore, the increased p38 phosphorylation persisted up to 4 h after initial exposure, unlike the TLR-ligands alone which were not significantly increased at 4 h in comparison to vehicle control (Figures 4.3A). Of note, no significant differences were observed for non-phosphorylated p38 for any of the TLR-ligands, alone or in combination with Mn, which suggests the observed increase in p38 activation was not associated with changes in protein expression for p38 (Figure 4.3A).

As we have also previously reported, p38 phosphorylation in response to LPS stimulation is elevated at 1 h, but diminishes by 4 h (Crittenden and Filipov, 2008). In this study, we examined p38 phosphorylation at 1 h for another TLR ligand, pI:C, which has differential signaling from that of LPS. Increased phosphorylation of p38 was observed at 1 h for pI:C-stimulated N9 microglia relative to vehicle, however it was less than Mn and pI:C in combination (Figure 4.3B). Similar to LPS alone as previously reported (Crittenden and Filipov, 2008), pI:C-induced p38 phosphorylation decreases by 4 h, suggesting that prolonged p38 phosphorylation is important for the enhanced

inflammatory cytokine production observed for the pI:C-Mn combination and may be important for Mn-potentiation of CPG- and GPG-induced TNF-α and IL-6 as well.

The relevance of the previous observations is underscored by the fact that each TLR (i.e. TLR2, TLR3, TLR4, and TLR9) utilizes somewhat different signaling pathways (Beutler, 2004; Takeda and Akira, 2004). In the case of pI:C, a distinctly different pathway is utilized, as pI:C-TLR3 signaling is not mediated by MyD88, but uses the TRIF adaptor molecule instead (Beutler, 2004; Takeda and Akira, 2004). Thus, the Mn-potentiation effect is not dependent on a specific signaling pathway. As Mn potentiates pI:C-induced TNF-α and IL-6, it seems likely that Mn-potentiation of TLR-induced inflammatory cytokine production is independent of MyD88. However, as CpG- and GPG-signaling occurs through TLR9 and TLR2 (respectively), and since both of these TLRs are believed to require the adaptor molecule MyD88 (Beutler, 2004), Mn-potentiation may utilize either MyD88 or TRIF.

Another likely target for Mn-potentiation of microglial inflammatory cytokines is the family of 'downstream' signaling kinases, p38, ERK, and JNK. We have previously demonstrated that pharmacological inhibition of p38, and to a much lesser degree ERK, prevented enhanced inflammatory cytokine production in Mn+LPS-exposed N9 microglia (Crittenden and Filipov, 2008). Additionally, research by Chen *et al.* (2006) reported not only Mn-potentiation of TNF-α in LPS/IFN-γ-stimulated primary mixed glia and increased phosphorylation of multiple (p38, ERK, JNK) kinases, but also that pharmacological inhibition of p38 prevented the potentiating effect of Mn. .

While our previous data regarding p38 activation is in agreement with others (Chen *et al*., 2006), we have not extensively examined the role(s) of ERK or JNK. It has

been reported that inhibition of ERK by UO126 was effective at preventing Mn-potentiation (Chen *et al*., 2006). This is in contrast to our previous studies using the ERK inhibitor, PD98059, which was not as effective as the p38 inhibitor SB203580 at preventing Mn-potentiation. As the ERK inhibitor PD98059 prevents activation of the inactive MKK 'upstream' of ERK, but not the activated MKK (Favata *et al.*, 1998), it is possible that incomplete inhibition of the MKK responsible for phosphorylating ERK may have underplayed the role of ERK in IL-6 and TNF-α production in Mn+LPS-exposed cells. To that end, using the ERK inhibitor UO126 that is reported to inhibit both inactive and active MKK (Favata *et al.*, 1998) may provide more information regarding the role of ERK in Mn-potentiation of TLR-induced cytokine production. Additionally, although it has been demonstrated that the JNK inhibitor SP600125 can prevent inflammatory cytokine synthesis in cells of the innate immune system, we have not previously examined this role of the JNK signaling pathway in Mn-potentiation of TNF-α and IL-6 by TLR-stimulated N9 microglia (Waetzig *et al.*, 2005; Zhou *et al.*, 2007).

To determine if 'partial' ERK inhibition may have been responsible for the incomplete inhibition of Mn-potentiation that we previously observed in our experimental model, as well as to examine the role of JNK, we exposed N9 microglia to inhibitors for p38, JNK, and ERK prior to exposure to Mn and/or TLR-ligands (LPS, pI:C, CpG, or GPG). Although all MAPK inhibitors (SB203580, SP600125, and UO126) were effective at preventing Mn-potentiation of TNF-α or IL-6, we observed a differential effect of MAPK inhibition among the TLR tested. Inhibition of Mn-potentiation for TNF-α and IL-6 in TLR2, TLR4, and TLR9-stimulated cells was greatest for the JNK

and ERK inhibitors, in contrast to TLR3 where p38 and JNK inhibition was most effective.

For TLR4 (LPS), this is somewhat in contrast to our previous work, but in agreement with Chen *et al.* (2006) who reported that prior exposure to SB203580, UO126, or SP600125 inhibited LPS- and Mn+LPS+IFN-γ-induced TNF-α production by mixed glia equally (Chen *et al.*, 2006). In the case of ERK, this may be due to the differences in the mechanism of action of the MAPK inhibitors (PD98059 vs. UO126). Although the role of the MAPK in Mn-potentiation of TLR2 and TLR9-induced inflammatory cytokines had not been previously examined, the inhibition of Mn-potentiated, TLR (CpG or GPG)-induced TNF-α and IL-6 expression in microglia pre-incubated with p38, JNK, or ERK inhibitors is not unprecedented, as the TLR2, TLR4, and TLR9 receptors use a similar signaling pathway (i.e. MyD88-dependent pathway; Takeda and Akira, 2004; Beutler, 2004). It is possible that inhibiting a target 'downstream' of MyD88 in one of these TLR pathways (e.g. TLR4) would have a comparable effect on a 'similar' TLR-signaling pathway (e.g. TLR2 or TLR9).

On the other hand, inhibition of JNK and ERK in CpG- or GPG-stimulated cells was more effective at preventing cytokine expression than p38 inhibition, as inhibition of JNK and ERK almost completely inhibited TNF-α and greatly reduced IL-6. This was not the case with TNF-α and IL-6 production in cells exposed to SB203580 followed by Mn+TLR (i.e. LPS, CpG, GPG), in that cytokine production was significantly inhibited, but not as much as with SP60125 and UO126. This could be explained by the observation that both TLR2 and TLR9 require MyD88 for intracellular signaling, while TLR4 can initiate intracellular signaling by a MyD88-dependent or –independent

pathway (Beutler, 2004; Takeda and Akira, 2004). It may be that MyD88-mediated signaling by TLR2 and TLR4 activation primarily utilizes JNK and ERK. Toll-like receptor 4-signaling, on the other hand, can initiate signaling by two different pathways, which increases the diversity of 'downstream' targets (Barton and Medzhitov, 2003; Beutler, 2004). Thus, TNF-α production in TLR4-stimulated cells is less dependent on individual intermediate signaling molecules (i.e. p38, JNK, or ERK).

Interestingly, Mn-potentiation of TLR3 (pI:C)-induced TNF-α and IL-6 is most sensitive to inhibition of p38 and JNK. As TLR3 is believed to be MyD88-independent, it may be that this signaling pathway primarily uses p38 and JNK to mediate intracellular signaling (Takeda and Akira, 2004; Beutler, 2004). However, as was observed overall for TLR2, TLR4, and TLR9-stimulated cells, inhibition of all three MAPKs was at least partially effective in preventing enhanced inflammatory cytokine production.

In summary, Mn-potentiation of TLR-induced inflammatory cytokines is not limited to LPS-stimulated microglia, and Mn-potentiation can occur for a broad array of TLR (i.e. pI:C, CpG, and GPG). Manganese-potentiation is not MyD88-dependent, as TNF-α and IL-6 production is potentiated in TLR3 (pI:C)-stimulated microglia, which is a MyD88-independent signaling pathway. Prolonged p38 phosphorylation is associated with Mn-potentiation in TLR (LPS, pI:C, CpG, GPG)-stimulated N9 microglia. Pharmacological inhibition of p38, ERK, and JNK inhibited inflammatory cytokine production in all TLR (LPS, pI:C, CpG, GPG)-stimulated cells, suggesting one or more of the MAPK are involved in TLR-mediated TNF-α and IL-6 production, as well as in the Mn-potentiation of these cytokines.

CHAPTER V

CONCLUSIONS

One of the fundamental biological tenets is the interaction of different cell types within the multicellular milieu of tissues and organs. The exchange of information in the form of neurotransmitters, hormones, cytokines and many other biomolecules, in appropriate concentrations and duration, allows cells to promote growth and development within the tissue and between organs, thereby promoting the fitness of the organism. However, excessive or prolonged expression of these molecules, as observed with inflammation, may be detrimental to cells and tissues, promoting cellular damage and death. Interestingly, the latter concept is similar to a fundamental tenet of toxicology, as the difference between a cure and a poison is attributed to the dose a chemical (reviewed in Ames and Gold, 2000). In the current study, we have attempted to address how exposure to a toxicologically relevant metal, such as Mn, results in enhanced and prolonged expression of inflammatory cytokines, with an emphasis on intracellular signaling, as this may provide insight into the mechanism of action, as well as potential targets for therapeutic intervention in cases of excessive Mn exposure.

Although it has been reported that Mn alone can induce mild to moderate expression of inflammatory mediators in non-activated glia (Bae *et al.*, 2006; Zhang *et al.*, 2007), the greatest effect has been observed in LPS- or LPS/INF-γ-activated glia. Indeed, production of cytokines and NO in LPS-activated microglia is potentiated by Mn,

typically resulting in 2-fold or more increases in inflammatory mediators relative to LPS alone (Barhoumi *et al.*, 2004; Filipov *et al.*, 2005; Crittenden and Filipov, 2008). However, whether Mn-potentiation is dependent on activation of the TLR4-signaling pathway, or a specific molecular component, is not clear. To demonstrate that Mn-potentiation is not limited to LPS-induced activation, we have shown that Mn-potentiation of microglial cytokine production can occur in cells activated by pI:C, CpG, or GPG, ligands for TLR3, TLR2, and TLR9, respectively (Figure 4.1). Additionally, we have demonstrated that the potentiation is not dependent on time of exposure, as exposure to Mn 3 h before or after microglial activation will result in enhanced IL-6 and TNF-α production (Figure 2.1). Taken together, Mn-potentiation is not dependent on activation by a specific TLR ligand (i.e. LPS, pI:C, CpG, or GPG) and is not limited to a narrow window of exposure, suggesting that exposure to Mn could occur before or after exposure to various TLR ligands.

On the other hand, activation of the CNS inflammatory cells may not be dependent on direct stimulation of microglial TLR. It has been shown that peripheral inflammation can induce activation of cellular inflammation in the CNS (Perry *et al.*, 1998; Cunningham *et al.*, 2005). It is possible that induction of peripheral inflammation can trigger neuroinflammation and, if Mn exposure has occurred/is occurring as well, the result could be an enhanced and prolonged inflammatory cytokine production in the brain. Although an intriguing hypothesis, there is no published data to support this possibility at the present time.

As the first reports to describe the potentiation effect were in glia co-exposed to Mn and LPS, with or without IFN-γ, (Spranger *et al.*, 1998; Chang and Liu, 1999;

Barhoumi *et al.*, 2004), the TLR4 signaling pathway appeared to be the logical candidate for the site of action of Mn. Some of the early studies clearly demonstrated a role for transcription factors like NF-kB, as well as signaling kinases like the MAPK, which are both components of the TLR4 signaling pathway (Chang and Liu, 1999; Filipov *et al.*, 2005; Bae *et al.*, 2006; Crittenden and Filipov, 2008). Therefore, we initially examined two of the MAPK thought to be critical for production of inflammatory mediators by microglia, p38 and ERK (Koistinaho and Koistinaho, 2002). We investigated the role of p38 and ERK using pharmacological inhibitors specific for each of these MAPK, SB203580 and PD98059, respectively. From this work, we concluded that p38, and not ERK, was primarily responsible for the Mn-potentiation we had observed. This data was in general agreement with the perceived role of p38 in stress and inflammation, while ERK appeared not to be as important in the observed Mn-potentiation (Koistinaho and Koistinaho, 2002).

However, published work by others using mixed glia or primary microglia suggested that, besides p38, JNK and ERK were also involved in the production of inflammatory mediators by microglia (Bae *et al.*, 2006; Liao *et al.*, 2007; Zhang *et al.*, 2007). Since we had not previously examined the role of JNK in our experimental system, we decided to assess the role of JNK using pharmacological inhibition (i.e. SP600125), as well as re-examine the role of ERK using a different pharmacological inhibitor, UO126. Although our early investigations suggested that p38 was, perhaps, most involved in the observed Mn-potentiation, pharmacological inhibition of any of the three major MAPK prevented both TLR- and Mn+TLR-induced inflammatory cytokine expression. This work was somewhat in contrast to our previous findings with ERK,

which may be due to incomplete inhibition of ERK by PD98059 as it only targets inactive ERK, while UO126 inhibits both inactive and active ERK (Favata *et al.*, 1998). Additionally, both SB203580 (p38) and SP600125 (JNK) prevented Mn-potentiation in LPS-activated N9 microglia.

Taken together, pharmacological inhibition of the MAPK (i.e. p38, ERK, or JNK) will inhibit Mn-potentiation in TLR-activated N9 microglia. Although p38 may not be the only MAPK involved in the Mn-potentiation of inflammatory cytokines, activation of p38 and its downstream targets like the cytokine transcription factor TATA-binding protein (TBP), as well as activation of NF-kB, is necessary for TNF-α expression (Carter *et al.*, 1999). We have demonstrated that Mn exposure not only increases p38 activation (i.e. phosphorylation), but also increases p38 kinase activity in LPS-activated N9 microglia. Moreover, as p38 kinase activity is responsible for activating transcription factors necessary for cytokine synthesis such as TBP (Carter *et al.*, 1999), it is possible that enhanced p38 phosphorylation and kinase activity may result in increased inflammatory cytokine mRNA and protein expression. Indeed, we have observed Mn-dependent increases in TNF-α mRNA and protein expression in LPS-activated N9 microglia. However, it is not clear if Mn effects TBP activation and/or nuclear translocation, as we have not examined activation of TBP at this time.

On the other hand, the role of JNK and ERK cannot be overlooked, as these MAPK play a role in the expression of inflammatory mediators by activated microglia (Waetzig *et al.*, 2005; Wu *et al.*, 2007). Although our current data is in general agreement with the literature, it is not clear if one or more of the MAPK is of greater

importance than the others in the Mn-potentiation of inflammatory mediators we have observed.

Another important question is what is responsible for the enhanced and prolonged activation of p38, and perhaps the other MAPK? As activation of the MAPK is thought to be dependent on the upstream MAP2K, we have examined the MAP2K primarily responsible for activating p38, MEK-3/6. In addition, we have examined two other MAP2K that may be important in the activation of p38, as well as ERK and JNK, MEK-1/2 and MEK-4, respectively. Although MEK-3/6 activation was not significantly changed by the presence of Mn, we did observe a small but significant increase in the phosphorylation of MEK-1/2 and MEK-4 at 1 h that diminished by 4 h. It is possible that the enhanced p38 phosphorylation observed at 4 h is due, in part, to increased activation of MKK-1/2 and MKK-4. However, as MKK-3/6 is considered the primary MAP2K responsible for activating p38, and because increased activation of MKK-1/2 or MKK-4 is not observed at 4 h, enhanced MAP2K activity may not be primarily responsible for the prolonged activation of TLR-activated, Mn-exposed microglia. An alternative to MAP2K activation of p38 is 'autophosphorylation', which requires p38 to associate with the TAK1/TAB1 complex (Ge *et al.*, 2002). However, we have not examined this possibility within our experimental model.

Another mechanism whereby Mn can prolong the activation of p38 is by acting on the phosphatase MKP-1. This may be important in the context of Mn-potentiation, as decreased MKP-1 was observed at 1 h in Mn-exposed cells. However, by 4 h the levels of MKP-1 mRNA and protein had significantly increased relative to controls. Typically, MKP-1 expression coincides with p38 activation, to serve as feedback control, thereby

preventing prolonged activity of p38 and enhanced expression of inflammatory mediators. In fact, we did observe increased MKP-1 expression at 1 h in LPS-exposed cells; however the magnitude of the increase was decreased substantially in cells exposed to Mn and LPS. This is in contrast to N9 microglia exposed to Mn, which had less MKP-1 mRNA and protein expression. Taken together, the initial decrease in MKP-1 expression could result in enhanced activation of p38. Furthermore, the delay in 'normal' feedback inhibition may result in increased MKP-1 at a later time point, as the signaling pathway within the cell works to deactivate p38. Indeed, this is what we observed at 4 h for MKP-1 in Mn-exposed cells. Although the decreased MKP-1 mRNA and protein in cells exposed to Mn may result in prolonged activation of p38, further examination of MKP-1 activation and enzymatic activity is necessary to determine if decreased MKP-1 expression is responsible for the persistent activation of p38.

In contrast to the role of the MAPK in Mn-potentiation of IL-6 and TNF-α, specific TLR activation is not required for enhanced inflammatory cytokine in the presence of Mn. This is relevant as activation of TLR2, TLR4, and TLR9 are dependent on the adaptor molecule MyD88, while MyD88-independent signaling via TLR3 requires TRIF. Thus, potentiation of inflammatory cytokines by Mn in TLR-activated microglia is not dependent on MyD88 activation. It may be that Mn acts 'downstream' of the initial TLR signaling pathway, suggesting that the MAPK, or another signaling pathway(s) intersecting with the MAPK, may be the molecular site of action.

On the other hand, Mn-potentiation of inflammatory cytokine protein may not be due to increased mRNA expression alone. Activation of the p38 MAPK signaling pathway not only increases inflammatory cytokine gene expression, but may also

promote mRNA stability, resulting in an increased half-life of the cytokine mRNA (Sun *et al.*, 2007). Indeed, p38 phosphorylation of downstream targets like MAPK activated kinase-2 (MK-2), as well as tristetraprolin (TTP), is necessary for preventing TNF-α mRNA degradation (Sun *et al.*, 2007). The enhanced p38 activation observed in Mn-exposed N9 microglia may increase cytokine protein by not only increasing mRNA expression, but also by increasing cytokine message stability.

In summary, (i) Mn-potentiation of inflammatory cytokine expression is not dependent on microglial activation by a specific TLR, or the TLR adaptor protein MyD88, (ii) activation of downstream signaling targets such as p38, ERK, and JNK appear to be necessary for enhanced cytokine mRNA and protein expression and (iii) increased and prolonged p38 activation partly due to the effects of Mn on MKK-1/2, MKK-4, and, importantly, MKP-1, is associated with the potentiating properties of Mn. Further examination of the MAPK cascade may provide important data in regards to Mn-potentiation of IL-6 and TNF-α. Specifically, examining alternative p38 activation mechanisms (i.e. autophosphorylation), as well as the activation of ERK and JNK, may indicate the molecular target for Mn-induced inflammatory cytokine potentiation. Additionally, examination of the molecular targets of p38, and perhaps ERK and JNK, may indicate whether the Mn-potentiation of inflammatory cytokines in TLR-activated microglia is mainly due to enhanced cytokine mRNA expression and/or to increased cytokine mRNA stability as well.

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