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Neuroinflammatory mechanisms and translational approaches in environmental neurotoxicity models

Colleen Elizabeth Jeffrey
Iowa State University

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**Neuroinflammatory mechanisms and translational approaches in
environmental neurotoxicity models**

by

Colleen Jeffrey

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Toxicology

Program of Study Committee:
Anumantha G. Kanthasamy, Major Professor
Arthi Kanthasamy
Bryan Bellaire
Dusan Palic

Iowa State University

Ames, Iowa

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ABSTRACT

Parkinson's disease (PD) is the most common neurodegenerative movement disorder first characterized in 1917 by James Parkinson, who called it the "shaking palsy". The pathogenesis of PD has not yet been elucidated, as it is a complex and multifactorial disease. PD is characterized by the progressive and selective loss of dopaminergic neurons within the substantia nigra. Both motor and non-motor symptoms are seen in patients with PD, including bradykinesia, rigidity, postural instability, depression, olfactory deficits, and dementia. Idiopathic PD, which accounts for approximately 95% of known PD cases, is age-related and affects approximately 3% of people over the age of 65. The pathology of PD was once believed to be simple, involving selective degeneration of the nigrostriatal pathway leading to the reduction of the nigrostriatal region's dopamine concentration. But the pathology of PD has been found to be much more complex, with evidence of degeneration of nondopaminergic transmitter systems as well as dopaminergic neurons. Over the years, new pathogenic mechanisms have been studied and implicated in the pathology of PD, including neuroinflammation, reactive microgliosis, oxidative stress, and mitochondrial dysfunction.

Recent studies evaluating exposure to transition metals have shown that manganese (Mn), by itself or in combination with an inflammatory stimulus, can activate pathways, leading to a neuroinflammatory response within the nigrostriatal dopaminergic system. Our lab has previously shown that the novel PKC isoform PKC δ contributes to manganese-induced apoptosis. When catalytically cleaved, PKC δ

activates caspase-3, which consequently activates caspase-9, leading to the activation of an apoptotic cascade. Previous studies by other laboratories showed that Mn can augment neuroinflammatory responses, including LPS-induced neuroinflammatory responses. We sought to investigate if PKC δ plays a role in LPS/Mn-induced inflammatory events using *in vitro* models of neuroinflammation. We showed increases in nitric oxide release, gp91phox protein expression, and the production of intracellular reactive oxygen species upon Mn/LPS treatment, which could be attenuated when cells were pretreated with the PKC δ inhibitor rottlerin. We also used primary microglia obtained from both wild-type mice and PKC δ knockout mice as a cell culture model of neuroinflammation. The primary microglia were treated with Mn/LPS and we saw a marked decrease in the release of nitric oxide, production of intracellular reactive oxygen species, and the release of various cytokines (IL-1 β , IL-6, IL-10, IL-12, and TNF α) in the PKC δ (-/-) microglia as compared to the microglia obtained from wild-type mice. Collectively, our results suggest that the PKC δ signaling pathway may play a key role in regulating key proinflammatory events, including microglial activation, cytokine release, and NADPH oxidase complex activity induced by LPS/manganese treatment.

Our lab also studied the novel compound diapocynin, a metabolite and dimer of the naturally occurring NADPH oxidase inhibitor apocynin, and its ability to attenuate LPS/Mn-induced neuroinflammatory events. Initially, we compared apocynin and diapocynin side-by-side to determine if diapocynin could effectively block neuroinflammatory events. We found that diapocynin had an EC₅₀ about eight times

lower than apocynin, and could more efficiently attenuate LPS-induced neuroinflammatory events including nitric oxide production, production of intracellular reactive oxygen species, and the release of various cytokines. Thus, we sought to determine if diapocynin was also able to attenuate neuroinflammatory events induced upon treatment with a combination of LPS and manganese. Using microglial cell culture models of neuroinflammation, we showed that diapocynin pretreatment effectively attenuated the production of nitric oxide and intracellular reactive oxygen species, and was able to suppress the release of $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-10 , and IL-12 . Diapocynin pretreatment was also able to reduce the expression of the phox protein gp91phox, a major component of the NADPH oxidase complex. Collectively, these results suggest that diapocynin can effectively suppress Mn/LPS-induced neuroinflammatory events.

Collectively, we have discovered that $\text{PKC}\delta$ may be a potential therapeutic target in neuroinflammatory events. As determined using $\text{PKC}\delta$ knockout microglia, the kinase plays a key role in augmenting metal-induced proinflammatory events in the brain. Additionally, we characterized the anti-inflammatory effects of diapocynin, a metabolite of apocynin. After establishing diapocynin as a more effective anti-inflammatory compound than apocynin, we characterized diapocynin as an effective suppressor of Mn/LPS-induced microglia inflammatory responses. Overall, the work presented in this thesis has important implications for development of novel therapeutic strategies for Parkinson's disease.

CHAPTER 1: GENERAL INTRODUCTION

Thesis Layout & Organization

The alternative format was used for this thesis. It consists of an overall abstract, a general introduction, three research papers, a general conclusion, references, and acknowledgments. The abstract introduces the research project discussed in this thesis. The general introduction chapter contains a brief overview of idiopathic Parkinson's disease. The background and literature review section introduces reactive microgliosis and current evidence demonstrating the role it plays in Parkinson's disease. It also reviews what is currently known about protein kinase C delta (PKC δ) and the role this protein plays in dopaminergic neuronal degeneration and neuroinflammatory processes. Also the review will outline what is known about diapocynin and current uses against inflammatory events. The background and literature review section will end with the research objectives pertaining to chapters II, III, and VI. Chapters II, III, and IV are research papers, which will be communicated to different journals. References for each of these chapters will be at the end of the corresponding chapter. The fifth chapter will be general conclusions pertaining to the findings in the previous chapters. References used for the introduction, background and literature review, and general conclusions sections will be in the final references section. The final section will be acknowledgements.

All of the research in this thesis described by the author was obtained during the course of her graduate studies at Iowa State University under the guidance and

supervision of his major professor and principal investigator Dr. Anumantha G. Kanthasamy.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and the most common neurodegenerative movement disorder and was first described by James Parkinson in 1817 as the "shaking palsy". The disease is characterized by the progressive and selective loss of dopaminergic neurons within the substantia nigra. PD is characterized by a variety of motor deficits including bradykinesia, akinesia, rigidity, and postural instability, as well as various non-motor symptoms ranging from depression and sleep disorders to dementia. This disease affects approximately 1 million people in the United States and more the 4 million worldwide (von Bohlen und Halbach, Schober et al. 2004). Idiopathic PD, which occurs in 95% of PD cases (Tanner 2003), is age-related and affects approximately 3% of people of the age of 65 (Whitton 2007).

The pathology of PD was often considered simple, involving the nigrostriatal pathway selective degeneration resulting in the reduction of dopamine concentration within the nigrostriatal region. Unfortunately, the pathology of PD is much more complex, and includes degeneration of nondopaminergic transmitter systems as well (Lang and Obeso 2004). As neurodegenerative research branches into new areas, various pathogenic mechanisms have been implicated in PD: neuroinflammation, reactive microgliosis, ER and oxidative stress, mitochondrial dysfunction, and apoptosis (Zhang, Perry et al. 1999; Mosley, Benner et al. 2006; Tansey, McCoy et al. 2007; Drechsel and Patel 2008). The diagnosis of PD has classically coincided with the onset of motor dysfunction manifestations. Increasing evidence lends support to the belief

that non-motor symptoms antedate the presentation of motor symptoms, including olfactory dysfunction, depression, sleep abnormalities, and constipation (O'Sullivan, Williams et al. 2008). Research of the past decades has shown new insight into the world of PD, but it has not, however, led to the development of a single coherent mechanism of nigrostriatal dopaminergic neuronal degeneration.

A newly emerging hypothesis has gained popularity amongst researchers: the 'multiple hit hypothesis' (Carvey, Punati et al. 2006; Sulzer 2007). Various studies have implicated complex interactions between environmental factors, genetics, and cellular dysfunctions as the underlying cause of selective neuronal death seen in PD (Warner and Schapira 2003; Obeso, Rodriguez-Oroz et al. 2010). This hypothesis stems from the belief that a variety of neurotoxins/neurotoxicants bombard the nigrostriatal system, possibly accumulating over time, exacerbating any intrinsic cellular dysfunction, and exploiting underlying genetic factors, leading to the onset of sporadic PD.

Environmental neurotoxicants have gained increasing interest as major risk causative factors in the development of sporadic PD. After a large twin study failed to support the hypothesis that genetic factors play a significant role in sporadic PD's etiology (Tanner, Ottman et al. 1999), non-genetic risk factors have become a target in PD research. Pesticides, inflammatory agents, and heavy metals have been implicated as possible risk factors through experimental and epidemiological studies (Di Monte, Lavasani et al. 2002; Olanow 2004; Cicchetti, Drouin-Ouellet et al. 2009; Burbulla and Kruger 2011).

As no single event can sufficiently explain PD's characteristic loss of dopaminergic neurons, chronic neuroinflammation and reactive microgliosis have gained interest as a possible mechanism to explain this neuronal degeneration (Block, Zecca et al. 2007; Whitton 2007; Hirsch and Hunot 2009). Post-mortem studies provided evidence of neuroinflammation in PD patient brains, with the presence of activated microglia in the area of the substantia nigra, cytokine accumulation, and oxidative damage to proteins in the brain (Hirsch and Hunot 2009; Tansey and Goldberg 2010). It is the sustained inflammatory response that may contribute to the progressive neuronal degeneration through an imbalance of pro- and anti-inflammatory responses leading to neurotoxic oxidative stress and cytokine-dependent toxicity (Hirsch and Hunot 2009).

Though current research has implicated a variety of processes and factors in the pathogenesis of PD, a coherent mechanism has not yet been elucidated. The multifactorial nature of PD pathogenesis makes it difficult to create therapies that can protect against the variety of factors that contribute to the development of the disease. A viable therapy would have to address multiple signaling pathways, and the complex etiology of PD.

Background and Literature Review

Neuroinflammation Introduction

Chronic neuroinflammation has become a ubiquitous finding in both patients and experimental models of PD, with similar evidence seen in other neurodegenerative diseases: Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Streit, Mrazek et al. 2004; Whitton 2007). The brain's innate immune system is comprised of glial cells, namely the microglia and astrocytes, though microglia are thought to be the main contributors to neuroinflammation (Streit and Kincaid-Colton 1995; Whitton 2007). Microglia, the innate brain macrophage, can become activated by a variety of triggers: pathogens, infection, and trauma, as well as other innate immune cells within the brain. Though inflammation is an organism's normal response, some types or modes of inflammation can be damaging, leading to deviations from CNS homeostatic conditions, which can create toxic environments for neurons leading to degeneration. During acute, short-lived circumstances, inflammatory mechanisms help to prevent injury while promoting healing. When these inflammatory mechanisms become chronically sustained, neuroinflammation can lead to damage of the surrounding host tissue. This makes the role of microglia dichotomous, as they contribute both to neuroprotection as well as neurodegeneration. The neuroinflammatory response is characterized by the release of cytokines and chemokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Liu, Gao et al. 2003; Block and Hong 2007). Many of these factors produced by microglia are proinflammatory and cytotoxic in

nature, and can cause the microenvironment surrounding the neurons to become toxic, leading to neuronal degeneration.

Neuroinflammation activates the neuroimmune system, and it is through the neuroimmune response and the subsequent release of a number of cell signaling molecules, that neurodegeneration occurs. When neuronal damage leads to the loss of communication between neurons and microglia, microglia reach a hyperactive state, due to being outside of neuronal control. It is believed that this is the point at which persistent neuroinflammation is reached, and present neuropathologies are exacerbated (Polazzi and Contestabile 2002).

Microglia activation and reactive microgliosis

Microglia play an important role in neuronal development, neuronal homeostasis, and neuronal survival (Streit 2002; Bessis, Bechade et al. 2007; Ransohoff and Perry 2009). Microglia are continually active, constantly surveying and scrutinizing the surrounding microenvironment. Any disturbance in the homeostasis of the nervous system elicits a phenotypic response from the microglia, constituting an activated microglia, based upon morphology and/or the expression of antigens on the cell surface (Ransohoff and Perry 2009). Morphological changes are induced by a variety molecules, including lipopolysaccharide (LPS), as seen in Figure 1 (Gordon, Hogan et al. 2011). Though morphological changes represent an activated microglia, even microglia in a “resting” state are highly dynamic, continuously changing their structure in

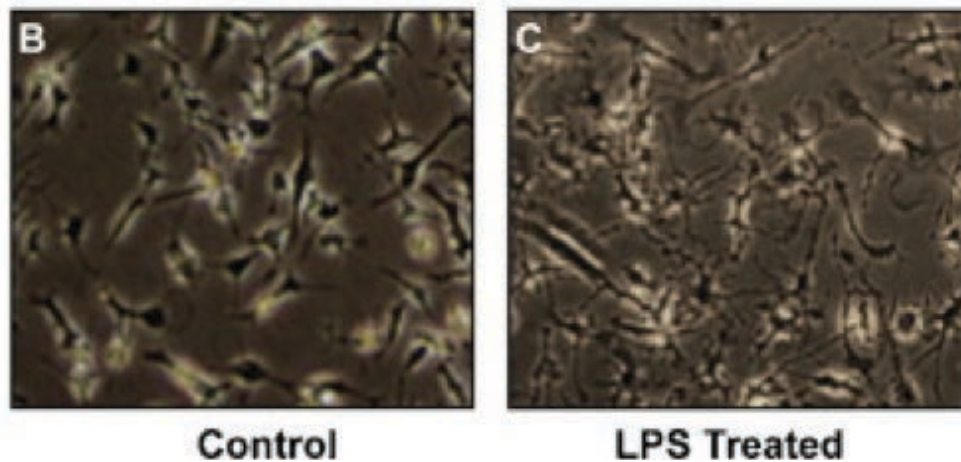


Figure 1: Morphological changes in microglia can be induced through LPS treatment.

response to the ever-changing surrounding environment. These changes are presumed to mediate microglial immune surveillance functions (Nimmerjahn, Kirchhoff et al. 2005).

Microglia are the resident macrophage for the brain and spinal cord, though the phenotype is distinct from macrophages found in tissue outside of the central nervous system (CNS). Microglia originate in the bone marrow, being derived from myeloid cells, and migrate to the brain during early development. The origin of microglia was conclusively confirmed by McKercher and group using PU.1 – null mice, which were absent microglia (McKercher, Torbett et al. 1996). The brains of these mice were repopulated by microglia once a bone marrow transplant was performed (Beers, Henkel et al. 2006). Microglia show regional diversity within the brain both numerically speaking and by immune receptor expression differences (de Haas, Boddeke et al.

2008). The regions of the CNS having the highest density of microglia are the hippocampus, substantia nigra, basal ganglia, and olfactory telencephalon.

Microglia are intimately connected with other cells in their immediate surroundings, both the environment and the expression of receptor-ligand pairs on neighboring cells, and the microglia themselves, will have a strong effect on microglia phenotype (Ransohoff and Perry 2009). A variety of ligands, signaling molecules, receptor expression, and so on determine the state of inactivation of microglia as well as microglia morphology. However, it is important to note that microglia do not go from resting to activated, but proceed from a surveillant state to effector microglia through various modes of progression. Microglia are able to show an early and rapid response to acute neuronal injury or insult, varying cell surface proteins and receptors.

The CNS is considered immune privileged in that it is self-regulated, with peripheral immune cells crossing an intact blood brain barrier (BBB), active macrophage and lymphocyte regulation by neurons and glia, and immunocompetent microglia directing lymphocytic neuroprotective responses (Carson, Doose et al. 2006; Harry and Kraft 2008). Beyond isolation from the peripheral immune system, the CNS immune privilege includes the ability of the neurons and glial to regulate the immune responses within the CNS. Though all cell types within the CNS are responsible for immune mediated responses, microglia are poised for rapid responses to environmental changes. Able to realign themselves in seconds or minutes and readily produce stimulus-specific factors and begin transcription of response genes giving rise to functional proteins within hours, microglia are extremely reactive. Microglia can both

upregulate an array of surface receptors and manufacture a variety of secreted factors. These secreted factors include pro- and anti-inflammatory cytokines and chemokines, nitric oxide, reactive oxygen species (ROS), growth factors, and glutamate (Harry and Kraft 2008). As with most dynamic processes, microglia activation is very tightly regulated, relying on crosstalk with neurons, astrocytes, and the extracellular background (Carson, Doose et al. 2006).

As mentioned, when activated, microglia can release an array of factors in response to the ever-changing environment. Many of these factors are essential to neuronal survival, including BDNF, GDNF, TGF β 1, and IL10 (Trapp, Wujek et al. 2007; Ekdahl, Kokaia et al. 2009). Microglia upregulation of GLT-1 has also been shown to be neuroprotective, shielding neurons from excitotoxicity (Persson, Brantefjord et al. 2005).

Microglia activation and reactive microgliosis play an important role in both neuroprotection and inflammation-mediated neurotoxicity, playing a dual role within the CNS. Because the primary role of microglia is neuroprotection, it is difficult to decipher when chronic inflammation, which leads to neurotoxic environments, begins, and therefore microglia's deleterious effect on neuronal health. All aspects of microglia activation are necessary for maintaining CNS homeostasis.

Evidence supporting a role for microglia activation in neurodegenerative diseases

McGeer *et al.* was the first researcher to suggest the involvement of inflammation in PD after illustrating MHC upregulation in PD patients as well as the presence of activated microglia within the substantia nigra (McGeer, Itagaki et al. 1988). Since these findings in 1988, various postmortem studies using brains from PD patients have shown extensive microglial activation within the nigrostriatal system (Whitton 2007; Hirsch and Hunot 2009). Hunot and Hirsch both described the presence of activated microglia expressing pro-inflammatory cytokines as well as inducible nitric oxide (iNOS) and reactive oxygen species (ROS), all of which modulate neuronal cell death in PD (Hunot, Boissiere et al. 1996; Hirsch, Hunot et al. 1998; Hirsch and Hunot 2009). Using PET scans, microglial activation was also measured in living PD patients, idiopathic PD, showing increased activation in the pons, basal ganglia, and frontal and temporal cortices (Gerhard, Pavese et al. 2006). Interestingly, the increased microglial activation was apparent in all PD patients, as compared to the healthy controls, and was not correlated to the patient's time since PD onset. Furthermore, epidemiological studies have also implicated inflammation in the etiology of PD, illustrating the ability of continual non-steroidal anti-inflammatory drug (NSAID) use to lower the risk of developing PD by approximately 46% in humans. Animal studies were used to confirm this data (Chen, Zhang et al. 2003; Chen, Jacobs et al. 2005).

Support for neuroinflammation involvement in PD has also been strengthened through studies showing a link between infection and neurodegeneration. Systemic

infections tend to exacerbate neurodegenerative diseases, pointing towards microglial involvement. Studies have illustrated microglial activation can be induced by systemic infection in patients with multiple sclerosis (MS) (Perry 2004). In patients with Alzheimer's disease (AD) worsening periods of dementia were exhibited following infections, and deteriorating symptoms in patients suffering from MS (Cunningham, Wilcockson et al. 2005; Perry, Cunningham et al. 2007).

Mechanisms of microglial neurotoxicity

In the adult brain, microglia are mainly in their 'resting' state, characterized by a ramified morphology. As already discussed, microglia in their 'resting' state are far from static, constantly palpating the environment removing debris and responding to even the slightest change in their microenvironment. Microglia have a diverse selection of extracellular receptors in order to respond rapidly to a variety of insults. These receptors are for endotoxins, cytokines and chemokines, ATP, factors found within the serum, and mis-folded proteins. These signaling molecules can activate the microglia directly via toxins, pathogens, or endogenous proteins, or indirectly through damaged neuronal signaling. Even upon cessation of the initial insult, microglia activation can persist through positive feedback from dying neurons, as well as signals from surrounding glial cells, and self-perpetuating cycles.

Microglia release a variety of inflammatory cytokines including tumor necrosis factor (TNF α), interleukin-1 β (IL-1 β), and IL-6, which have the ability to amplify and sustain inflammatory responses. The levels of these three cytokines are shown to be

increased in the basal ganglia and CSF of PD patients (McGeer, Yasojima et al. 2001). Inflammatory cytokines may be able to mediate harmful inflammation by two proposed mechanisms; direct activation via surface receptor binding activation on dopaminergic neurons or indirectly through glial-cell activation and subsequent expression of inflammatory factors (Hirsch and Hunot 2009).

Microglia are also capable of causing cell toxicity through the production and release of toxic oxygen-derived and nitrogen-derived products via respiratory or oxidative burst/stress. Several enzymatic systems contribute to the production of superoxide and nitric oxide free radicals, namely NADPH oxidase and inducible NOS. Interestingly, within the substantia nigra of PD patients the expression of these biocatalytic systems is increased (Hunot, Boissiere et al. 1996; Wu, Teismann et al. 2003). The free radicals produced, NO and O_2^- , are known to be poorly reactive, but combine to form peroxynitrite ($ONOO^-$), a highly reactive nitrogen species able cause oxidative damage in a variety of proteins including tyrosine hydroxylase and α -synuclein (Ara, Przedborski et al. 1998; Przedborski, Chen et al. 2001; Hirsch and Hunot 2009). Figure 2 outlines how these mechanisms can perpetuate neuroinflammation and reactive microgliosis, propelling a cycle the continues uncontrolled and further potentiates neuronal cell death (Glass, Saijo et al. 2010).

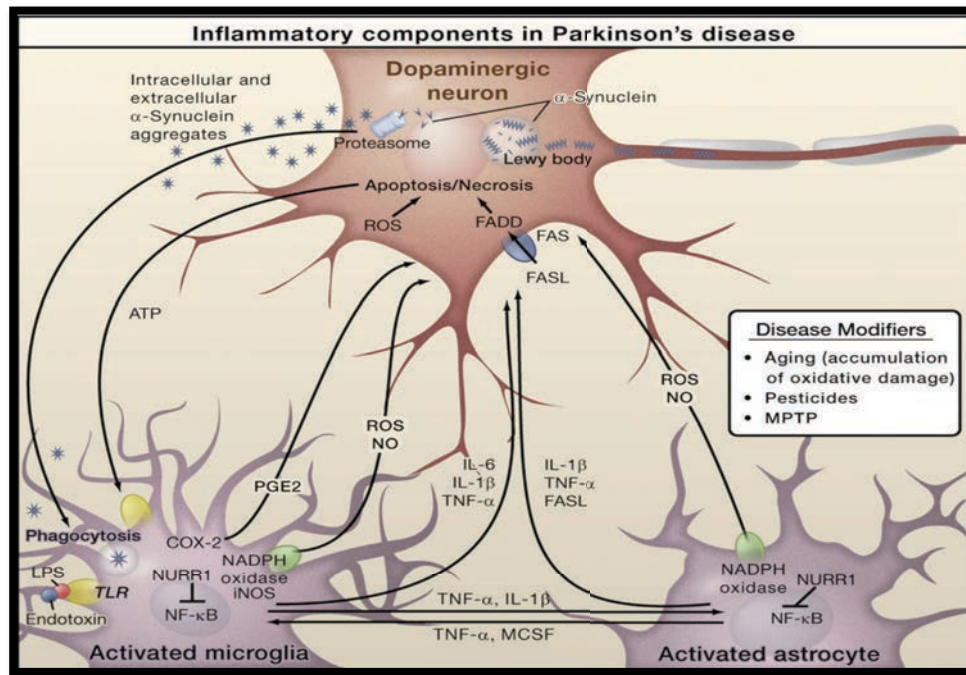


Figure 2: Illustration of neuroinflammatory mechanisms associated with neurodegeneration (Glass, Saijo et al. 2010).

Mechanisms of LPS-induced neuroinflammation in cell culture models

The use of the bacterial endotoxin LPS has given great insight into the role of neuroinflammation perpetuated by microglial activation in the pathophysiology and progression of PD. LPS has been used in both cell culture and animal models to lend further support to the idea that sustained neuroinflammation can lead to neurotoxic environments and subsequent neuronal cell death aiding in the progression of neurodegenerative diseases. LPS has been shown to be a potent microglia activator and has been used in numerous mechanistic studies over the past decades (Zielasek and

Hartung 1996; Suzumura, Takeuchi et al. 2006; Qin, Wu et al. 2007; Henry, Huang et al. 2009; Svensson, Fernaeus et al. 2010; Tansey and Goldberg 2010).

LPS has been shown to cause morphological changes within microglia, inducing their activated amoeboid appearance, as opposed to their 'resting' state ramified appearance (abd-el-Basset and Fedoroff 1995). LPS treatment also induces production of various pro-inflammatory factors including IL1 β , IL6, TNF α , ROS, and RNS (Lee, Liu et al. 1993; Righi 1993; Blasi, Barluzzi et al. 1995). All of these phenomena are classic characteristics of inflammation, lending support to the concept of using LPS to study the mechanisms of neuroinflammation.

LPS binds to toll-like receptor 4 (TLR4), in turn activating various pathways: IKKs, PI3K, MAP3Ks, and IRF3. Activation of the TLR4 receptor promotes the secretion of pro-inflammatory cytokines as well as activating certain genes that are necessary for initiating adaptive immune responses. NF κ B, one of the transcription factors activated through TLR4 activated pathways, plays a key role in immune response regulation upon infection insult, and subsequent regulation of genes responsible for both the innate and the adaptive immune response (Gilmore 2006). A second transcription factor activated upon LPS stimulation is IRF3, a member of the interferon regulatory transcription factors. IRF3 also plays a pivotal role in an organisms innate immune response upon viral infection (Collins, Noyce et al. 2004).

Protein kinase C-delta (PKC δ) and its role in neurodegeneration

PKC δ is a member of the novel PKC family thus making it a calcium independent isoform, and is ubiquitously expressed throughout an organism's tissues (Leibersperger, Gschwendt et al. 1991). PKC δ can be activated through at least three different mechanisms: nuclear translocation, proteolytic cleavage, and phosphorylation of a tyrosine residue. This is an oxidative stress-sensitive kinase and plays a key role in apoptotic cell death in PD (Anantharam, Kitazawa et al. 2002; Kaul, Anantharam et al. 2005).

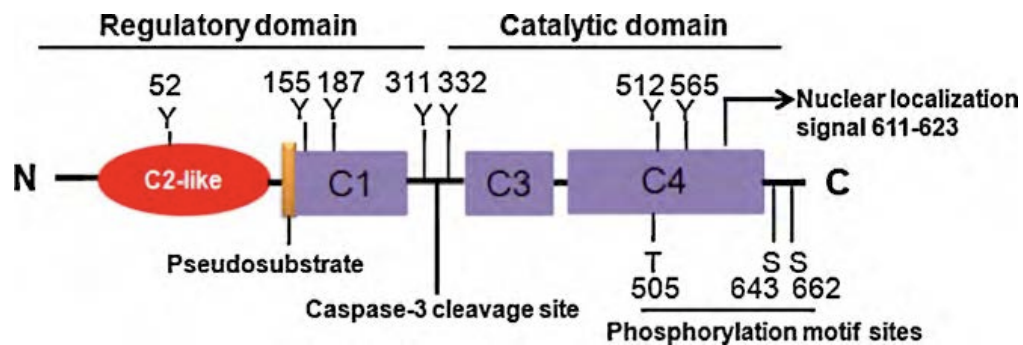


Figure 3: Schematic of PKC δ (Kanthasamy, Jin et al. 2010)

Recent studies have demonstrated PKC δ is highly expressed in the nigral tissue of mice. It also colocalizes with tyrosine hydroxylase (TH), the rate-limiting enzyme necessary for the production of DOPA, the precursor to dopamine (Zhang, Kanthasamy et al. 2007). These studies also outline another relationship between this kinase and the nigrostriatal pathway, showing PKC δ negatively regulates TH activity and dopamine synthesis. Kanthasamy *et al.* show a schematic of PKC δ 's structure in Figure 3.

The nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) complex

Reactive oxygen species (ROS) are considered an inevitable byproduct of aerobic metabolism, typically, though there are enzymes which function as producers of ROS. The NADPH oxidase complex is one such enzyme, and is expressed in microglia, among other cells within the CNS (Bedard and Krause 2007). ROS has become known as a major contributor to damage within biological organisms, avidly interacting with a variety of molecules including proteins, nucleic acids, carbohydrates, and lipids (Bedard and Krause 2007). There are different isoforms of the enzyme deemed NOX1 – NOX5, all of which are transmembrane, reducing oxygen to superoxide by transporting electrons across membranes. NOX2, also known as gp91phox, is one of the most extensively studied NOX isoforms, and is referred to as the phagocyte NADPH oxidase, showing phagocyte-specific tissue expression (Sumimoto, Miyano et al. 2005). As microglia have phagocytic tendencies, the focus and discussion will be on NOX2, as the NADPH oxidase complex.

In order to create superoxide, electrons are first transferred from NADPH to FAD. This first step is regulated by the activation domain of p67phox, one of the cytosolic subunits of the NADPH oxidase complex (Nisimoto, Motalebi et al. 1999). A single electron is then transferred to the iron center of the heme group, which can only accept one electron, forcing it to donate its electron to the outer heme so that the inner heme group can accept the second electron, from the initial FADH₂ molecule. Oxygen

must then be bound to the outer heme electron in order to create an energetically favorable state, as the transfer of the electron from the inner heme group to the outer heme group is energetically unfavorable (Doussiere, Gaillard et al. 1996; Cross and Segal 2004).

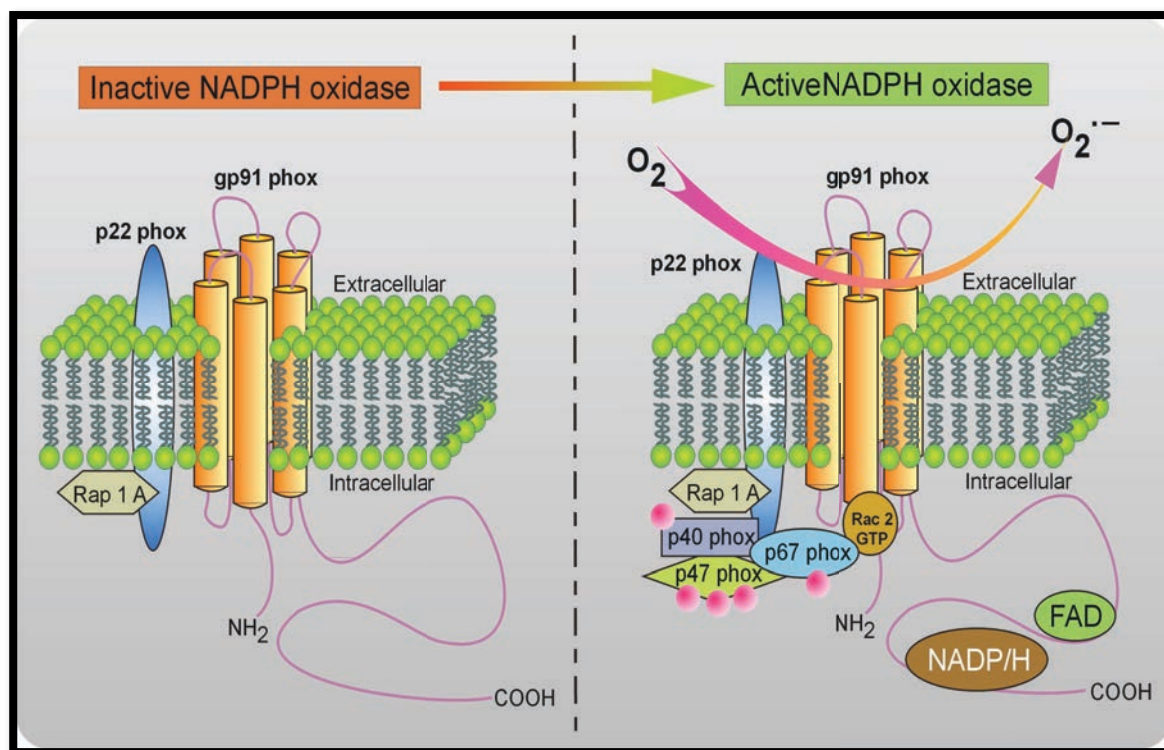


Figure 4: Schematic of the NADPH oxidase structure in inactive and active form.
(http://www.genkyotex.com/img_cms/Dessin%20NADPH%20oxydase.jpg)

When there is an imbalance between ROS synthesis and antioxidants a phenomenon known as oxidative stress, which can be extremely deleterious to surrounding cells, is reached. Therefore, the NADPH oxidase enzyme represents a potential therapeutic target as treatment for various diseases. Apocynin (4-hydroxy-3-methoxyacetophenone) is isolated from the roots of *Apocynum cannabinum* or Canadian

hemp, and is an efficient NADPH oxidase inhibitor (Stefanska and Pawliczak 2008). Though the mechanism of inhibition is not completely understood, it is known that the translocation of p47phox, a cytosolic subunit, is impaired and this subunit is unable to migrate to the membrane and activate the NOX2 complex (Peters, Hiltermann et al. 2001; Barbieri, Cavalca et al. 2004).

Environmental factors and neurodegenerative diseases

Upon discovering MPTP, it became clear that some neurotoxicants can reproduce both the neurochemical and pathological features associated with idiopathic Parkinsonism, through the specific targeting of the nigrostriatal system. MPTP was discovered after a group of intravenous drug users unknowingly injected themselves with a synthetic analog of Demerol, contaminated with MPTP. These individuals developed parkinsonism-like symptoms that were alleviated with levodopa treatment (Langston, Ballard et al. 1983). Since this occurrence, the search for environmental risk factors began, and correlations between certain neurotoxicants, lifestyles, and Parkinson's disease came to light.

Epidemiological studies implicate a number of environmental risk factors that may increase an individual's risk of developing Parkinson's disease. The risk factors include agrochemicals (pesticides, herbicides, etc.) (Liu, Gao et al. 2003), well-water consumption, industrial chemicals, certain occupations (e.g. mining, farming, welding), and heavy metal exposure (Tanner and Langston 1990; Gorell, Johnson et al. 1998;

Elbaz, Clavel et al. 2009) and have all been associated with increases in neurodegeneration.

Table 1: Environmental factors that may play a role in the development of neurodegenerative diseases.

Toxicant	Authors (Year)	Toxicant	Authors (Year)
Paraquat	Liou et al. (1997)	Maneb	Uversky (2004)
	Tanner et al. (1990) Semchuck et al. (1991)	Organophosphates	Costa et al. (2008) Bhatt et al. (1999)
Disulfide-based fumigants	Chapman et al. (1991)	Manganese	Gorell et al. (1999) Aschner et al (2007) Dobson et al (2004) Latchoumycandane et al. (2007)
Fungicides	Klaasseen, ed. (2001) Costa (2006) Meco et al. (1994)		
Organochlorines	Seidler et al. (1996)	Mercury	Ngim et al. (1989) Monnet-Tschudi et al. (2006)
Chlorophenoxy	Semchuck et al. (1993)		
Thiocarbamates	Semchuck et al. (1992)	Lead	Monnet-Tschudi et al. (2006)
Carbamates	Seidler et al. (1996)	Vanadium	Afeseh-Ngwa et al (2009)
Rotenone	Uversky (2004)	Zinc	Negga et al. (2011)
	Wang et al. (2006) Cannon et al. (2009)	Copper	Gorell et al. (1997)

Paraquat, a pesticide used extensively throughout the world, has been used as a PD model. It is believed that paraquat's underlying mechanism is related to ROS, whether directly or indirectly (Berry, La Vecchia et al. 2010), as well as its ability to activate caspase-3, which can lead to apoptosis (Peng, Mao et al. 2004). Maneb, a fungicide, is able to cross the blood brain barrier. Its mechanism of action is poorly understood, but it is known that it preferentially inhibits mitochondrial complex III (Zhang, Fitsanakis et al. 2003). Rotenone, a broad-spectrum insecticide, has the ability

to inhibit mitochondrial complex I and has been shown to induce the aggregation of α -synuclein and lead to the formation of Lewy bodies (Uversky 2004; Cicchetti, Drouin-Ouellet et al. 2009). Rotenone has also been shown to activate microglia and, at high concentrations, be directly neurotoxic to neuron-enriched cultures, void of microglia (Gao, Hong et al. 2002).

Idiopathic PD and metal toxicity has mostly revolved around the transition metals and their potential role in neurodegenerative diseases. Earle (1968) first reported the finding of increased iron in PD patient brains, with subsequent studies showing this accumulation occurs in the pars compacta of the substantia nigra (Earle 1968; Sofic, Riederer et al. 1988; Dexter, Wells et al. 1989). Iron toxicity may be achieved through increased accumulation coupled with decreases in its binding capability, along with the possibility of increases in the concentration of hydroxyl radicals via the Fenton reaction, assuming both iron and hydrogen peroxide are available, leading to oxidative stress (Di Monte, Lavasani et al. 2002). From the identification of iron as a potential factor in neurodegeneration, other metals have been studied for their possible role in neurodegenerative disease progression.

Manganese and neurodegeneration

Manganese (Mn), an essential trace element, plays an important role in a number of biological processes. Manganese functions as an essential cofactor for a variety of enzymes critical for both metabolic and redox homeostasis within the CNS. Glycosyltransferase, pyruvate decarboxylase, glutamine synthetase, and superoxide

dismutase are all dependent upon manganese to function properly (Gonzalez-Zulueta, Ensz et al. 1998; Keen, Ensunsa et al. 2000; Takeda 2003). Excessive exposure to Mn, however, has been known to lead to accumulation within the striatum, globus pallidus, and the substantia nigra resulting in a neurodegenerative disorder referred to as manganism (Milatovic, Zaja-Milatovic et al. 2009). Epidemiological studies have implicated a causal relationship between the risk for Parkinsonian-like symptoms and increased environmental Mn exposure, especially due to certain workplace environments such as welding, smelting, and mining (Aschner, Guilarte et al. 2007; Zhang, Wong et al. 2009).

When the brain is exposed to Mn, it can be taken up by astrocytes and neurons, the role for microglia in Mn toxicity is poorly understood, the astrocytes acting as the main storage site (Aschner 1999; Aschner, Vrana et al. 1999). This increase in Mn concentration, however, can cause an alteration in the release of certain factors, which may elicit an excitatory neurotoxic response (Erikson and Aschner 2003). Additionally, increases in iNOS, ROS, proinflammatory prostaglandins, and inflammatory mediators are seen upon Mn-induced neurotoxicity (Liu, Gao et al. 2002; Erikson and Aschner 2003; Erikson, Dorman et al. 2007).

Postmortem studies of human brains have shown Mn-induced neuronal damage to be prominent in the striatum as well as other structures of the basal ganglia, but preferential targeting of the dopaminergic neurons within the pars compacta is thought to be spared (Perl and Olanow 2007). Manganism is also associated with striatal dopaminergic neuron integrity alterations, causing decreases in dopamine transport

function and/or dopamine levels within the striatum (Milatovic, Zaja-Milatovic et al. 2009). Collectively, these results lead researchers to believe Mn-induced neurotoxicity is associated with oxidative damage, free radical production, neuroinflammation, and an alteration of the striatal dopaminergic neuronal integrity.

Recently, researchers have been investigating Mn modulation of LPS-induced inflammatory responses, especially focusing on the involvement of microglia. Various groups have demonstrated the ability of Mn to enhance TNF α , IL6, and NO production by microglia activated with LPS (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006), as well as IL1 β and ROS (Zhang, Lokuta et al. 2010). These findings lend support to the 'multiple hit hypothesis', showing that multiple factors play a role in the etiology of neuroinflammatory disorders, and can in fact synergistically interact to increase incidence of these diseases.

CHAPTER II: A ROLE FOR PROTEIN KINASE CDELTA (PKC δ) IN LPS/MN-INDUCED NEUROINFLAMMATION

Abstract

Exposure to environmental neurotoxicants such as metals and pesticides have been shown to trigger dopaminergic degeneration; therefore, these chemicals are considered potential risk factors of idiopathic Parkinson's disease (PD). Neuroinflammation has gained increasing recognition as a critical mechanism for sustained dopaminergic degeneration, and thus a contributing factor in the progression of PD. Recent studies have shown that exposure to manganese (Mn), by itself or in combination with inflammatory stimuli, can activate neuroinflammatory responses in the nigrostriatal dopaminergic system. We previously showed that novel PKC isoform PKC δ contributes to manganese-induced apoptosis. In the present study, we sought to investigate if PKC δ plays a role in LPS/Mn-induced inflammatory events. Low dose Mn exposure (30 μ M) in the BV2 microglial cell model potentiated LPS-induced iNOS activation as measured by nitrite release. Also gp91phox protein, a vital component of NADPH oxidase complex, is activated, as evident from increased protein expression as well as increased production of intracellular reactive oxygen species upon Mn/LPS treatment. Pretreatment with PKC δ inhibitor rottlerin attenuated Mn/LPS-induced gp91phox protein expression increase, suggesting that PKC δ may have a role in Mn/LPS-induced activation of NADPH oxidase complex. Mn treatment in primary microglia obtained from PKC δ knockout (-/-) mice showed a marked decrease in the

levels of cytokines released (IL-1 β , IL-6, IL-10, IL-12, and TNF α) as compared to PKC δ (+/+) primary microglial cells. Collectively, these results suggest that the PKC δ signaling pathway may play an important role in regulating LPS/manganese-induced key proinflammatory events, including microglial activation, cytokine release, and NADPH oxidase complex activity.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by the progressive degeneration of nigral dopaminergic neurons and loss of striatal dopamine (Olanow and Tatton 1999). Neuroinflammatory insult mediated by sustained microglia activation has been increasingly implicated in the pathogenesis of PD, along with other neurodegenerative diseases. Throughout the past decade, compelling studies, including human, primate, and animal, along with epidemiological data, show neuroinflammation as a self-perpetuating pathogenic process contributing to the progression of PD (Whitton 2007).

Microglia are the resident immune cells in the brain, playing a key role in immune surveillance and injury repair (Zhang, Wong et al. 2009). Exposure to transition metals such as manganese and iron has been linked to the pathogenesis of PD (Earle 1968; Di Monte, Lavasani et al. 2002). Notably, recent studies show Mn treatment can induce as well as augment the neuroinflammatory responses in both *in vitro* and *in vivo* models (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006; Moreno,

Sullivan et al. 2008). Research suggests that exposure to Mn or Mn-containing compounds, such as the fungicides Maneb and Mancozeb, can elicit various changes at the cellular level, including a depletion in both glutathione and dopamine levels, an increase in oxidative stress, and disruption in the antioxidant system (Kitazawa, Wagner et al. 2002; Roth and Garrick 2003; Olanow 2004). Excessive Mn exposure activates resident microglial cells and astrocytes, resulting in the release of a variety of proinflammatory cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS). However, the biochemical mechanisms underlying Mn-induced cytokine release and oxidative stress are not well understood.

We recently demonstrated that Protein Kinase C δ (PKC δ), a member of the novel PKC family, plays a key role in mediating manganese neurotoxicity (Kitazawa, Anantharam et al. 2005; Latchoumycandane, Anantharam et al. 2005). Mn has been shown to activate a specific cell death signaling event. Mn induces the release of cytochrome c from the mitochondria into the cell cytosol, leading to the activation of caspase-9, which in turn activates caspase-3. The activated caspase-3 mediates the proteolytic cleavage of PKC δ and proteolytically activated PKC δ aids in DNA fragmentation (Latchoumycandane, Anantharam et al. 2005).

Knowing that Mn can activate microglia, causing a neuroinflammatory response, we set out to determine if PKC δ plays a role in the LPS/Mn-induced neuroinflammatory events including cytokine release and oxidative/nitrosative stress.

Materials and Methods

Materials. Lipopolysaccharide and rottlerin were purchased from Calbiochem (San Diego, CA). Inorganic manganese was purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 and DMEM/F-12 medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). Bradford reagent for protein assays was purchased from Bio-Rad (Hercules, CA). The Griess reagent was purchased from Sigma (St. Louis, MO). The fluorescent ROS probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from EMD Chemicals (Philadelphia, PA). Primary antibodies utilized were gp91phox (mouse monoclonal, BD Transduction Laboratories, San Jose, CA), NOSII (rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β -actin (mouse monoclonal, Sigma, St. Louis, MO). Secondary antibodies, for Western blotting, used were IRDye 800-conjugated anti-rabbit (Rockland Labs, Gilbertsville, PA) and Alexa Fluor 680 conjugate anti-mouse (Licor, Lincoln, NE).

Cell cultures and treatment. BV2 microglia, an immortalized mouse microglial cell line obtained from Dr. Debomoy K. Lahiri (Indiana University School of Medicine, Department of Psychiatry, Indianapolis, IN), were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin and kept in a static environment maintained at 37°C with a humidified atmosphere containing 5% CO₂. Cells were treated in RPMI-1640 containing 2% FBS and pen/strep

with 1µg/ml LPS, 30µM Mn, and a combination of the two, along with a 30 minute pretreatment of rottlerin (1µM). Primary microglia from postnatal mouse pups were obtained through column-free magnetic separation outlined in our previous publication (Gordon, Hogan et al. 2011). Briefly, using the EasySep mouse CD11b positive selection kit from STEMCELL Technologies (Catalog #18770), magnetic separation of confluent mixed glial cultures was completed after 14-16 days of incubation. After media was aspirated from each T-75 flask, 3 ml of 0.25% trypsin was added and flasks were placed on a rotary shaker and allowed to agitate for 20 minutes. The trypsin reaction was stopped by adding 3 ml DMEM/F12 complete media, DMEM/F12 containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin, and a single cell suspension, using trituration, was obtained. Cells were then centrifuged for 6 min at 200 x g. The magnetic separation was performed according to the manufacturer's instruction save the final wash step, which was repeated 3 times in order to achieve a purity level of >97%. The purified microglia were grown in DMEM/F12 media containing 10% fetal bovine serum, 50 U/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine, 100 µM non-essential amino acids, and 2 mM sodium pyruvate. Cells were kept at 37°C in a humidified incubator with 5% CO₂. Cells were treated in DMEM/F12 complete media containing 2% FBS. Cells were treated with 100-500 ng/ml LPS, 30 µM Mn, or a combination of both, pretreatment of cells was with 1 µM rottlerin 30 minutes prior to treatment with LPS and Mn.

Nitric oxide detection. Supernatant nitric oxide (NO) concentration was determined indirectly through supernatant nitrite concentration quantification using the Griess reagent. BV2 cells were seeded at a concentration of 30,000 cells per well in 96-well plates and magnetically separated primary microglia were seeded onto poly-D-lysine coated 96-well plates at 200,000 cells per well. Both cells types were allowed to attach overnight before being treated in 125 μ l of medium. Upon the end of the treatment, 100 μ l of supernatant was pipetted from each well and placed in a new 96-well plate, to which was added 100 μ l of Griess reagent. The plate was incubated on a plate shaker for 10 minutes at room temperature, allowing a stable color to be obtained. Using a Synergy 2 multi-mode microplate reader (BioTek Instruments), the absorbance was read at 540 nm. A sodium nitrite standard curve was used to determine nitrite concentration for each sample.

Intracellular reactive oxygen species quantification (iROS). Production of intracellular reactive oxygen species (ROS) was determined using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), following previously published protocols (Zhang, Wang et al. 2005; Qian, Gao et al. 2007). Magnetically separated primary microglia were plated at 50,000 cells per well in poly-D-lysine coated 96-well plates. Cells were treated in DMEM/F-12 complete media for 6 and 24 hours, with pretreatment with rottlerin occurring 30 minutes before treatment. When the treatment ending time-point was reached, the medium was removed from the wells and the cells were washed with HBSS three times. HBSS containing 10 μ M DCF-DA was

added to each well (100 μ l) and allowed to incubate for 1 hour covered with foil in a humidified CO₂ incubator at 37°C. (Cells were only placed in the middle wells of the 96-well plate and HBSS was placed in surrounding empty wells to help prevent non-specific fluorescence). The intensity of the DCF-DA fluorescence was read at an excitation of 485/20 and an emission of 530/25, again using the Synergy-2 multi-mode microplate reader. The control fluorescent mean values were subtracted from the treatment groups fluorescent values, to remove background fluorescence, leaving the increase in fluorescence caused by the different treatments as an increase in iROS (Zhang, Wang et al. 2005; Qian, Gao et al. 2007).

Cytokine detection and quantification. Magnetically separated microglia were plated at 100,000 cells per well into poly-D-lysine coated 96-well plates. The microglia were allowed to attach overnight before being treated with 100-500 ng/ml LPS, 30 μ M Mn, or a cotreatment of LPS and Mn, and pretreatment with 1 μ M rottlerin 30 minutes prior to treatment with LPS+Mn. The treatment was allowed to continue for 24 hours, upon which time 50 μ l of supernatant was collected from each well and frozen and kept at -20°C until it could be analyzed. The Luminex bead-based immunoassay system was used to determine supernatant cytokine levels, and concentrations were extrapolated using recombinant cytokine standards (Vignali 2000; Gordon, Hogan et al. 2011).

Western Blotting. BV2 cells were seeded in T75 flasks and treated with 1 μ g/ml LPS, 30 μ M Mn, or a cotreatment of LPS and Mn. for 24 hours. Rottlerin, 1 μ M, was used as a

30-minute pretreatment before addition of LPS and Mn cotreatment. Cells were collected by scrapping after treatment. Lysates were prepared from BV2 cells using modified RIPA buffer (Pierce Biotechnology). Proteins were resolved using 10-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes overnight. Non-specific binding sites were blocked by Rockland blocking buffer for 1 hour. The membrane was incubated with anti-gp91phox (1:1000 dilution) or NOS2 (1:200 dilution) overnight at 4°C followed by secondary Alexa Fluor 680 conjugate anti-mouse or IRDye 800-conjugated anti-rabbit (1:10,000 dilution) antibodies for 1 hour at room temperature. β -actin (1:10,000) was probed for to determine equal protein loading in each lane. Antibody-bound proteins were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Data Analysis. Prism 4.0 software package (GraphPad Software, San Diego, CA) was used for all data analysis. Data was analyzed using the unpaired student t-test or initially analyzed by one-way ANOVA followed by Tukey's post-test, to compare all of the treatment groups. Statistically significant differences were considered if the p-value was less the 0.05.

Results

Determination of non-cytotoxic concentrations of manganese

In order to determine a working concentration of Mn, BV2 cells were exposed to various doses of Mn. After 24 hours the cells were viewed under a microscope to

determine if the Mn was toxic (Figure 1). At levels of 250 μM Mn or higher confluency of cells within these flasks was greatly decreased, indicating an environment that is toxic to the cells, either through cell death or an inability to replicate effectively. From this initial experiment and following what concentrations others have already worked with, a dose of 30 μM Mn was chosen as the working concentration.

Rottlerin inhibits LPS-induced iNOS activation

Rottlerin is a selective inhibitor of PKC δ (Gschwendt, Muller et al. 1994; Latchoumycandane, Anantharam et al. 2005), and was used to pharmacologically inhibit LPS/Mn-induced proinflammatory events in this study to determine the role PKC δ plays. To determine the working concentration of rottlerin to use, an EC₅₀ for rottlerin's ability to attenuate LPS-induced iNOS activation was constructed (Figure 2), and an EC₅₀ of 810 nm was determined, and a working concentration of 1 μM was used in subsequent studies.

Low dose Mn potentiates increases in iNOS activity in combination with LPS, rottlerin is able to attenuate iNOS activation in primary microglia

Previous studies have discussed a synergistic relationship between Mn and LPS in conjunction with NO production (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006). By quantifying the amount of nitrite found in the supernatant, we could indirectly determine NO production and corresponding iNOS activity. To ensure we could replicate the ability of Mn to potentiate LPS-induced iNOS activation, we first measure nitrite production in BV2 cells after a treatment time of 24 hours (Figure 3). We found LPS alone could increase iNOS activity by approximately 400%, while Mn alone, at 30

μM , did not cause a statistically significant increase in iNOS activation as compared to the control group. The combination of LPS + Mn, however, increased iNOS activation approximately 1500% above the control groups and 1000% above the LPS alone group. This data demonstrates the ability of Mn to potentiate LPS activation when considering nitrite production.

Furthermore, we repeated the study in primary microglia, which is a more indicative model of *in vivo* response. The trend of iNOS activation in the primary microglia mimicked the trend seen in the BV2 cell line (Figure 4). LPS alone increased supernatant nitrite levels from 0.1 μM to over 1 μM , while low dose Mn had no effect on iNOS activation as compared to the control group. The cotreatment of LPS + Mn further increased nitrite production to close to 2 μM , almost doubling the production seen in the LPS alone group. Rottlerin (the selective PKC δ inhibitor) was able to significantly attenuate the LPS/Mn-induced increase in iNOS activation, lending support to the hypothesis that PKC δ plays a role in LPS/Mn-induced NO production.

Rottlerin is able to attenuate LPS/Mn-induced increased protein expression of NOS2 and gp91phox

The protein NOS2, also called iNOS, is the enzyme responsible for the production of NO in conjunction with L-arginine (Dawson and Dawson 1998). To support the iNOS activation data gathered through indirect NO measuring by quantification of a main metabolite nitrite, we looked at the expression of the NOS2 protein in BV2 cells (Figure 5a). We see that the expression appears to be increased with LPS alone, while low dose Mn does not seem to have an affect on NOS2 protein expression. There is a significant

increase in NOS2 protein expression upon treatment with both LPS and Mn, following the relationship seen in the nitrite production assay. Rottlerin was able to bring NOS2 expression levels back down to basal levels, showing a role for PKC δ in iNOS activity.

As increased ROS production can be an extremely deleterious inflammatory event (Ara, Przedborski et al. 1998; Hirsch and Hunot 2009), we also looked at the protein expression of one of the NADPH oxidase complex subunits, gp91phox, in BV2 cells (Figure 5b). We saw a similar trend in gp91phox protein expression as seen in NOS2 protein expression. Using LPS we observe an increase in the gp91phox protein expression (though not statistically significantly), while low dose Mn had no such effect. An increase in gp91phox protein expression was observed upon LPS and Mn cotreatment. Rottlerin attenuated the LPS/Mn-induced increase in gp91phox protein expression, supporting the hypothesis that PKC δ plays a role in LPS/Mn-induced inflammatory events.

Rottlerin blocks LPS/Mn-induced cytokine production in primary microglia

As cytokines play an important role in modulating inflammation (Block, Zecca et al. 2007), we performed an experiment to determine if PKC δ plays a role in LPS/Mn-induced release of proinflammatory cytokines. We quantified the supernatant concentration of five cytokines: IL-1 β , IL-6, IL-10, IL-12, and TNF α (Figure 6). LPS and LPS/Mn induced a significant increase in all five cytokines, while Mn showed no increase in cytokine production. Rottlerin was able to significantly attenuate the LPS/Mn-induced cytokine production in four of the five cytokines. IL-1 β did not show

any attenuation. This data suggests that PKC δ plays a role in LPS/Mn-induced cytokine production and subsequent release.

Microglia cultured from PKC δ knockout animals show a significant decrease in iNOS activity as compared to microglia cultured from wild-type animals

We further solidified the idea that PKC δ plays a role in LPS/Mn-induced iNOS activity (indirectly measured through nitrite release) using an *ex vivo* model, obtaining microglia from wild-type and PKC δ knockout animals (Figure 7). LPS alone was only able to significantly increase nitrite production in the microglia cultured from the wild-type mice. In both cell types, PKC δ +/+ and PKC δ -/-, we observe the ability of Mn to potentiate LPS-induced iNOS activation through a significant increase in nitrite production. Though LPS and Mn were able to significantly increase nitrite production, as compared to the control group, in the absence of PKC δ , the increase was significantly lower than what was seen when PKC δ was present in the microglia. These results suggest the PKC δ plays a role in LPS/Mn-induced nitrite production, and, correspondingly, iNOS activity.

Microglia cultured from PKC δ knockout animals show a significant decrease in cytokine release as compared to microglia cultured from wild-type animals

In order to further solidify that PKC δ plays a role in LPS/Mn-induced cytokine release, we used microglia cultured from wild-type and PKC δ knockout animals (Figure 8). Here we see that release of all five cytokines is greatly inhibited in the absence of PKC δ . Using microglia from PKC δ knockout animals we see a significant decrease in the

release of IL-1 β , which we did not see in our earlier study when using rottlerin to block PKC δ activity.

Intracellular ROS production is inhibited in the absence of PKC δ

Emerging evidence shows augmentation of oxidative stress levels in the dopaminergic neuronal microenvironment is a pivotal factor in the degeneration of these neurons. These environmental changes can be caused by microglial neuroinflammatory responses. To determine if PKC δ plays a role in production of intracellular ROS (iROS), DCF-DA, a fluorescent probe, was used to quantify production (Zhang, Wang et al. 2005; Qian, Gao et al. 2007). In this instance, we observed the ability of both LPS and Mn (alone) to significantly increase the amount of iROS present in primary microglia (Figure 9). A further increase in iROS was seen when cells were treated with both LPS and Mn, which was almost completely attenuated upon rottlerin pretreatment (Figure 9a). Again, we used microglia cultured from PKC δ knockout and wild-type mice to further confirm a role for PKC δ in LPS/Mn-induced iROS production. The results show that iROS production was essentially completely blocked when PKC δ was absent (Figure 9b).

Discussion

In this study, we confirm that PKC δ plays a role in LPS/Mn-induced neuroinflammation and that Mn can potentiate certain LPS-induced microglial responses. We found that low dose Mn, alone, is not sufficient to elicit a complete proinflammatory response. Low doses of Mn in combination with LPS can, however,

elicit multiple proinflammatory responses including NO production, iROS production, and the release of various cytokines. Using rottlerin to pharmacologically inhibit PKC δ , we observe attenuation in supernatant nitrite, release of four proinflammatory cytokines, and the protein expression of NOS2 and gp91phox. To further substantiate that PKC δ plays a role in LPS/Mn-induced proinflammatory events, we repeated the studies using primary microglia cultured from PKC δ wild-type and knockout mice. We observed that iNOS activity, iROS production, and cytokine release were drastically inhibited in the absence of PKC δ . These results suggest that PKC δ plays a role in LPS/Mn-induced inflammation in microglia.

Manganese is an essential trace element, playing a pivotal role in a number of biological processes. Normal Mn concentrations within adult human tissue range from 3 to 20 μ M (Roth and Garrick 2003), with a mean brain Mn level of 0.261 μ g/g (Markesbery, Ehmann et al. 1984). Mn concentration differs within the brain dependent upon the region, the putamen and substantia nigra have Mn concentrations of 6.31 and 0.34 ng/mg wet weight, respectively (Zecca, Pietra et al. 1994).

When exposed to high amounts of Mn, an individual can develop a disorder known as manganism, which mimics many symptoms associated with Parkinson's disease. Mn has the ability to cross the blood brain barrier via specific carriers including transferrin and divalent metal transporter 1, as well as through simple diffusion (Yokel and Crossgrove 2004). Postmortem studies of PD patient brains show Mn-induced neuronal damage in structures of the basal ganglia, with prominent damage seen in the striatum, however, preferential targeting of pars compacta dopaminergic neurons,

which is seen in PD, seems to be spared (Perl and Olanow 2007). Though dopaminergic (DA) neurons within the pars compacta do not appear to be preferentially targeted, alteration in striatal dopaminergic neuron integrity has been observed, leading to decreases in dopamine transport function and/or striatal dopamine levels (Milatovic, Zaja-Milatovic et al. 2009). Mn-induced neurotoxicity is associated with oxidative damage, free radical production, and neuroinflammation, all of which have been implicated in the pathology of many neurodegenerative diseases, including Parkinson's disease.

Recent studies have investigated multiple insult theories, believing that sustained neuroinflammation and subsequent neurodegeneration is a result of the culmination of several factors leading to complex interactions between environmental factors, genetics, and cellular dysfunctions (Warner and Schapira 2003; Obeso, Rodriguez-Oroz et al. 2010). Filipov et al. (2005) and Chen et al. (2006) observed the ability of Mn to modulate LPS-induced inflammation, touching upon the idea that interactions between environmental factors can increase the risk for neuroinflammatory events. Though the mechanism through which LPS/Mn-induced neuroinflammation occurs is not well characterized, especially the role that microglia play.

Microglia are the resident immune cells of the brain, constantly surveying their environment, playing vital roles in both immune surveillance and injury repair (Kreutzberg 1996). Microglia are able to respond to toxicant-induced insults more quickly than astroglia, via a faster acting activation kinetic, as well as having the ability

to produce a wider variety of neurotoxic factors (Liu, Gao et al. 2003). These points suggest that microglia play a more prominent role, compared to astrocytes, in glial activation-mediated neurodegeneration (Liu, Gao et al. 2003).

Due to DA neuronal sensitivity to oxidative stress, ROS is considered particularly toxic (Jenner and Olanow 1998). When PKC δ was absent, we saw an almost complete inhibition of iROS, when using the microglia cultured from knockout mice (Figure 8b). Not only is a decrease in ROS important due to their deleterious effects on DA neurons, hydrogen peroxide, which is efficiently produced by microglia following Mn treatment (Zhang, Hatter et al. 2007), can react with iron to form cytotoxic oxidative radicals via the Fenton reaction. Iron is highly concentrated in the substantia nigra, a good portion of it bound to neuromelanin, which is thought to play a role in attenuating free radical damage, possibly through its ability to chelate transition metals (Gerlach, Double et al. 2003). Gerlach et al. (2003) also hypothesized that an increase in iron concentration (seen in PD patient substantia nigras) may saturate iron-chelating sites, leading to increases in iron and subsequent increases of free radical species. It stands to reason that decreases in ROS production would also be beneficial in that it would decrease the amount of hydrogen peroxide available to undergo the Fenton reaction, supporting PKC δ as a possible therapeutic target.

It is also important to mention the ability of ROS and RNS to react with each other to further increase the toxicity of an already cytotoxic environment (Hirsch and Hunot 2009). Superoxide anions can react with NO to produce the highly reactive nitrogen species peroxynitrite, which has the ability to cause oxidative damage in a

variety of proteins, including tyrosine hydroxylase and α -synuclein (Ara, Przedborski et al. 1998; Przedborski, Chen et al. 2001; Hirsch and Hunot 2009). The ability of proinflammatory factors to react with one another increases their neurotoxic potential, allowing for an increase in the deleterious risks they pose to surrounding cells.

Cytokines play an important role in the immune response, and microglia are able to produce a myriad of cytokines, quickly, following an insult. In our studies, we did not observe a Mn potentiation of LPS when quantifying cytokine release as other groups have discussed. We observed in the absence of PKC δ , LPS/Mn-induced cytokine release was significantly blocked (Fig. 8).

In summary, this study demonstrates that PKC δ may play a pivotal role in LPS/Mn-induced neuroinflammation. First, our studies show a synergistic relationship between LPS and Mn when observing iNOS activity. We have shown the ability of rottlerin, a selective PKC δ inhibitor, to attenuate LPS/Mn-induced iNOS activity, release of specific proinflammatory cytokines (IL-6, IL-12, and TNF α), and IL-10, as well as iROS in primary mouse microglia. We also show rottlerin is able to attenuate LPS/Mn mediated increased protein expression of NOS2 and gp91phox in BV2 cells. Using microglia obtained from PKC δ knockout mice, we were able to study differences in microglial response when PKC δ was absent. We found that in the absence of PKC δ LPS/Mn-induced neuroinflammatory factors were inhibited, as observed through iNOS activity, iROS production, and the release of specific cytokines (IL-1 β , IL-6, IL-10, IL-12, and TNF α). Collectively, these results indicate that PKC δ is a potential target for neuroinflammatory therapies.

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Figure Legends

Figure 1. Response of BV2 cells to different concentrations of manganese. BV2 cells were seeded into T-75 flasks and allowed to attach overnight. The cells were then treated with different concentrations of Mn to determine toxic concentrations of Mn becomes toxic to the cells. We observed that concentrations of up to 100 μM of Mn showed no visible signs of toxicity based upon cell confluency and appearance. As the concentration of Mn increased to 250 μM and above, cells showed signs of toxicity via detachment and decreases in confluency.

Figure 2. Rottlerin EC50 against LPS-induced iNOS activity. Using LPS to stimulate iNOS activity, an EC50 curve was constructed to determine the EC50 of rottlerin, in terms of its ability to attenuate LPS-induced iNOS activation. LPS was used at a concentration of 1 $\mu\text{g/ml}$, and the doses of rottlerin ranged from 50 nM to 5 μM (n=6). Supernatant nitrite concentration, using Griess reagent, was used to indirectly measure iNOS activity. The EC50 of rottlerin, in attenuating LPS-induced iNOS activity, was determined to be 810 nM.

Figure 3. LPS and Mn cotreatment can increase nitrite production in BV2 cells. BV2 cells were treated for 24-hours, before the supernatant was collected and nitrite concentration was determined (n=13). LPS alone was able to significantly increase iNOS activity, while Mn (30 μ M) had no significant effect on iNOS activity. The combination of both LPS and Mn significantly increased iNOS activity above the control group, as well as above the LPS treated group. These data illustrate that low dose Mn by itself does not elicit an increase in iNOS activity, but is able to potentiate the ability of LPS to increase iNOS activity in BV2 cells. (***) denotes a p-value < 0.001 as determined by one-way ANOVA).

Figure 4. LPS/Mn-induced nitrite production can be attenuated upon rottlerin pretreatment. Mn was also able to potentiate LPS-induced nitrite production. Rottlerin (Rott) was able to assuage some of the LPS/Mn-induced increase in nitrite production. Primary microglia were treated for 24-hours, using rottlerin as a 30-minute pretreatment (n=9, rott n=8). We observed a significant increase in nitrite production upon LPS and LPS+Mn treatment as compared to the control group. Mn treatment and rottlerin alone treatment, however, did not increase nitrite production. When Mn was used in conjunction with LPS it was able to potentiate the LPS-induced nitrite production increase. (** denotes a p-value < 0.01, *** denotes a p-value < 0.001 as determined by unpaired t-test).

Figure 5. Expression of NOS2 and gp91phox are increased with LPS/Mn treatment, which rottlerin is able to attenuate. BV2 cells were treated in T-75 flasks for 24-hours, using rottlerin as a 30-minute pretreatment in one group (n=3). Figure 5a. shows densitometric analysis of NOS2 protein production. It appears that LPS treatment increases expression of NOS2, while Mn has no effect on NOS2 protein expression. Using both LPS and Mn, we see a significant increase in the expression of NOS2, correlating well to our findings in iNOS activity. Rottlerin was able to return NOS2 protein expression levels back down to near basal levels. Figure 5b. shows densitometric analysis of gp91phox (a membrane bound subunit of the NADPH oxidase complex) protein expression. Following the trend seen with NOS2 protein expression, it appears LPS treatment increases gp91phox expression, though no significance was found statistically, while Mn treatment did not seem to effect gp91phox protein expression. LPS+Mn was able to significantly increase the expression of gp91phox, while rottlerin was able to significantly attenuate this increase in expression. (* denotes p-value < 0.05, ** denotes p-value < 0.01 as determined by unpaired t-test).

Figure 6. LPS/Mn treatment was able to increase cytokine release in primary microglia. Rottlerin was able to decrease LPS/Mn-induced increases in 4 of the 5 cytokines in the assay. Cells were treated for 24-hours, rottlerin was used as a 30-minute pretreatment. Five cytokines were probed for: TNF α , IL-6, IL-1 β , IL-10, and IL-12 (n=4). We observed that Mn did not increase cytokine production and release, while LPS/Mn caused a significant increase in cytokine release. Rottlerin was able to

significantly block release of four of the cytokines, it was not able to block the LPS/Mn-induced increase in IL-1 β release. (* and *** denote p-value < 0.05 and p-value < 0.001, respectively, as determined by one-way ANOVA).

Figure 7. An attenuation in nitrite production is seen in the absence of PKC δ using microglia cultured from wild-type and PKC δ knockout mice, treated for 24-hours (n=6). The PKC δ +/+ group shows a significant increase in nitrite production upon treatment with LPS, Mn, and LPS+Mn, as well as potentiation between LPS and Mn. In the PKC δ -/- group, we only see a significant increase in nitrite production in the LPS+Mn group, which is significantly lower than the amount of nitrite produced by PKC δ +/+ microglia undergoing the same treatment. (*, **, and *** denote p-values < 0.05, 0.01, and 0.001, respectively. All values determined using unpaired t-test).

Figure 8. Cytokine release is attenuated in the absence of PKC δ . Using microglia cultured from wild-type and PKC δ knockout mice, we were able to observe an inhibition in cytokine release in the absence of PKC δ . Both cell types were treated for 24-hours before the supernatant was collected and later assayed for cytokine concentration (n=2). Here, we observe that in the absence of PKC δ cytokine release is significantly blocked in all five cytokines for both LPS and LPS+Mn treatments. (***) denotes p-value < 0.001 as determined by one-way ANOVA).

Figure 9. Intracellular ROS production is attenuated in the absence of PKC δ . Using primary microglia we are able to observe intracellular ROS production (iROS). 9a. Shows iROS production can be significantly induced by LPS, Mn, and LPS+Mn (24-hour treatments). LPS/Mn-induced iROS production can be almost completely inhibited by a 30-minute rottlerin (rott) pretreatment (n=18, rott n=6). Rottlerin alone does not significantly affect iROS production (***) denotes p-value < 0.001 as determined by unpaired t-test). 9b. Using microglia obtained from wild-type and PKC δ knockout mice, we observe an almost complete inhibition in iROS production in the absence of PKC δ . (***) denotes a p-value < 0.001 between PKC δ +/+ and corresponding PKC δ -/- treatment group, as determined by unpaired t-test).

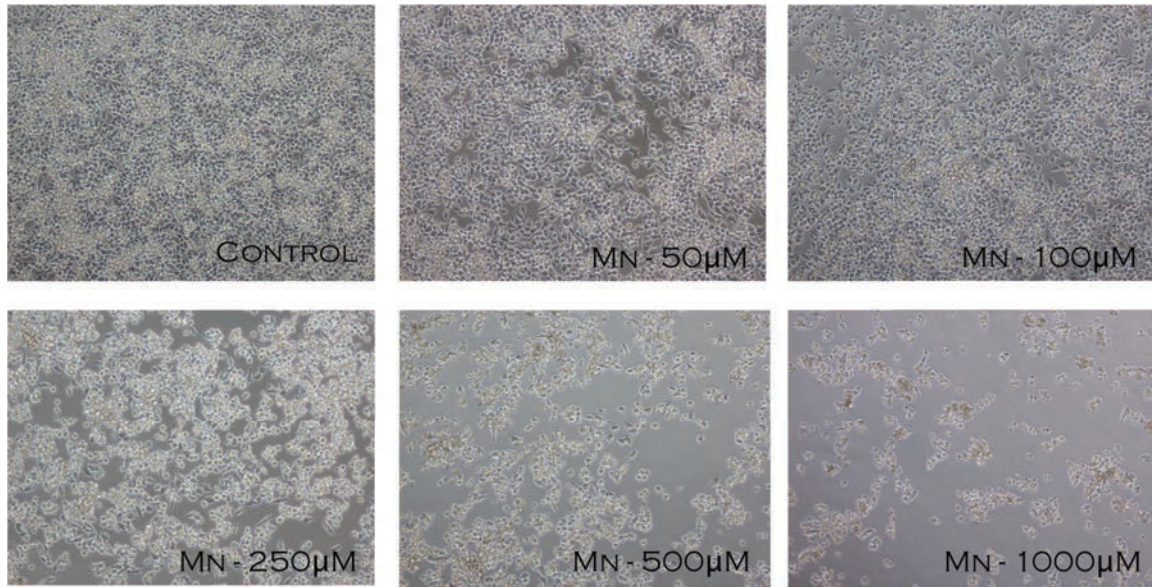
Figures

Figure 1. BV2 cells were treated with various doses of manganese to determine non-lethal doses.

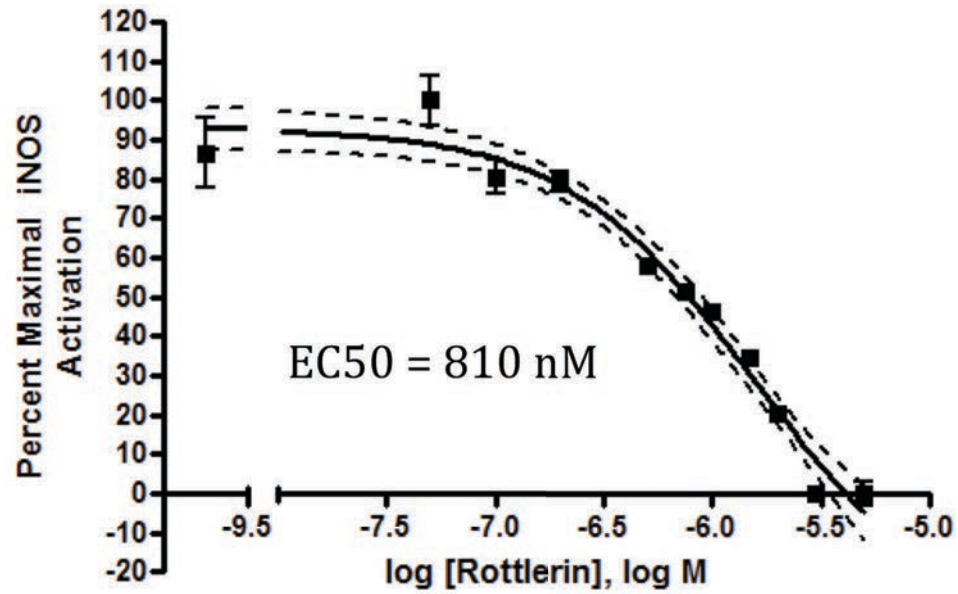


Figure 2. Rottlerin has an EC50 of 810 nM blocking LPS-induced iNOS activity.

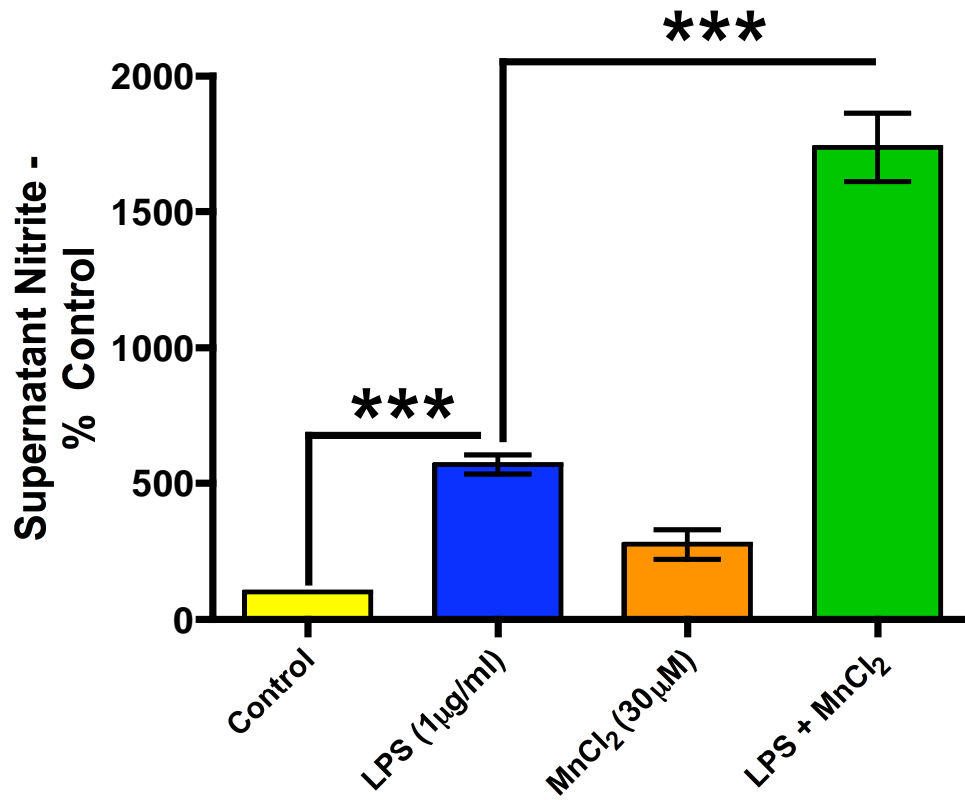


Figure 3. BV2 cell iNOS activity was determined through nitrite production.

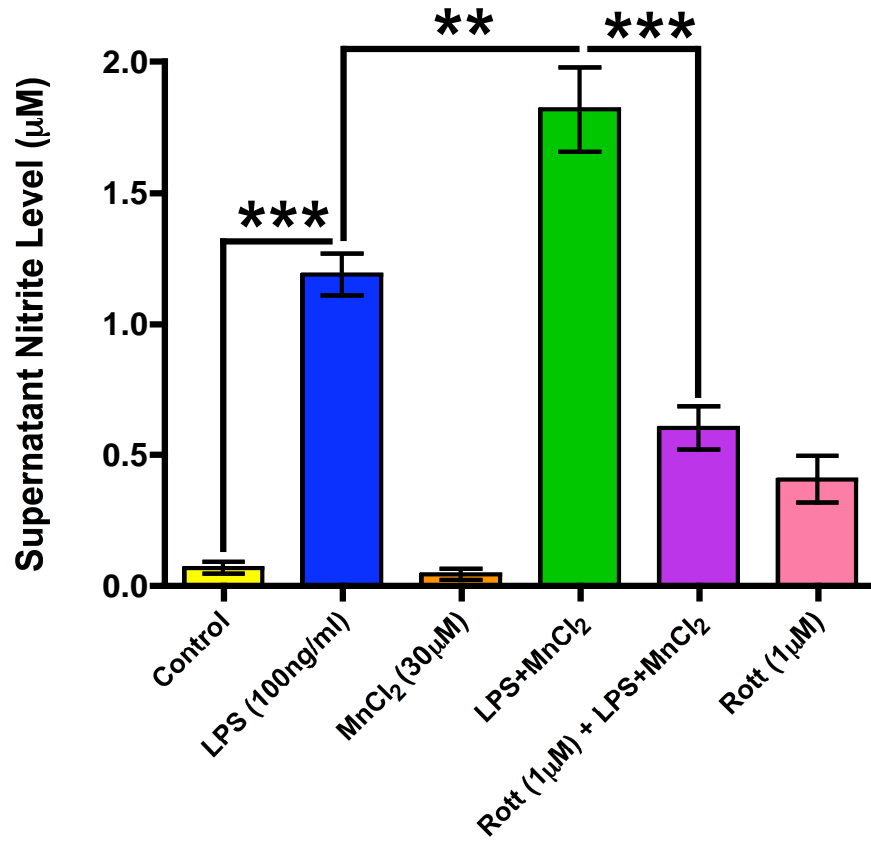


Figure 4. Primary microglia were treated for 24-hours, using rottlerin as a 30-minute pretreatment. Supernatant nitrite levels were determined using the Griess reagent.

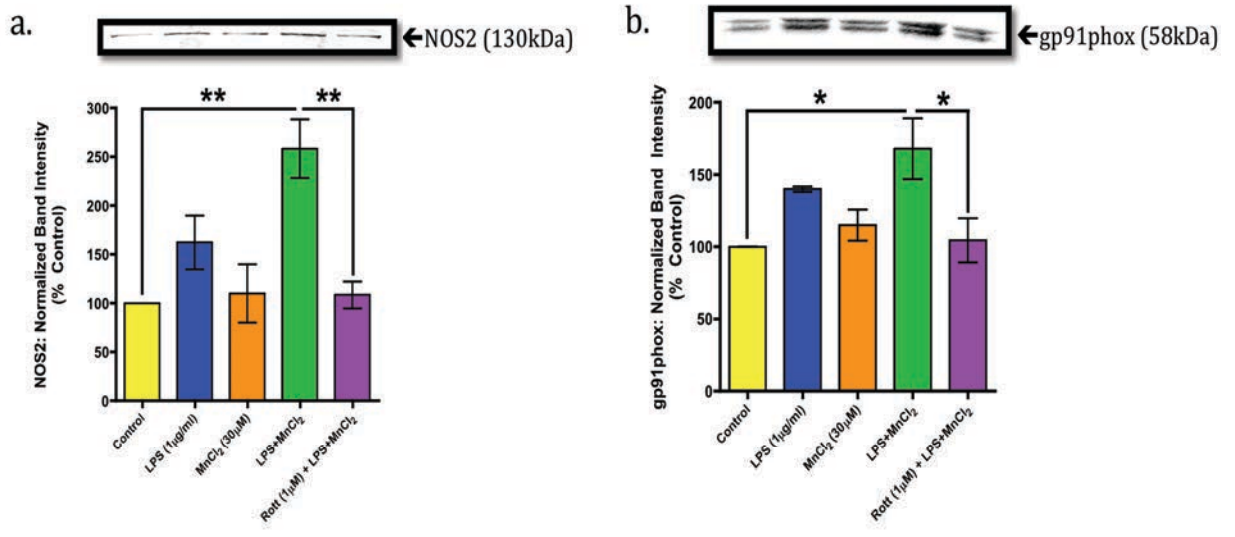


Figure 5. Protein expression of a. NOS2 and b. gp91phox in BV2 cells.

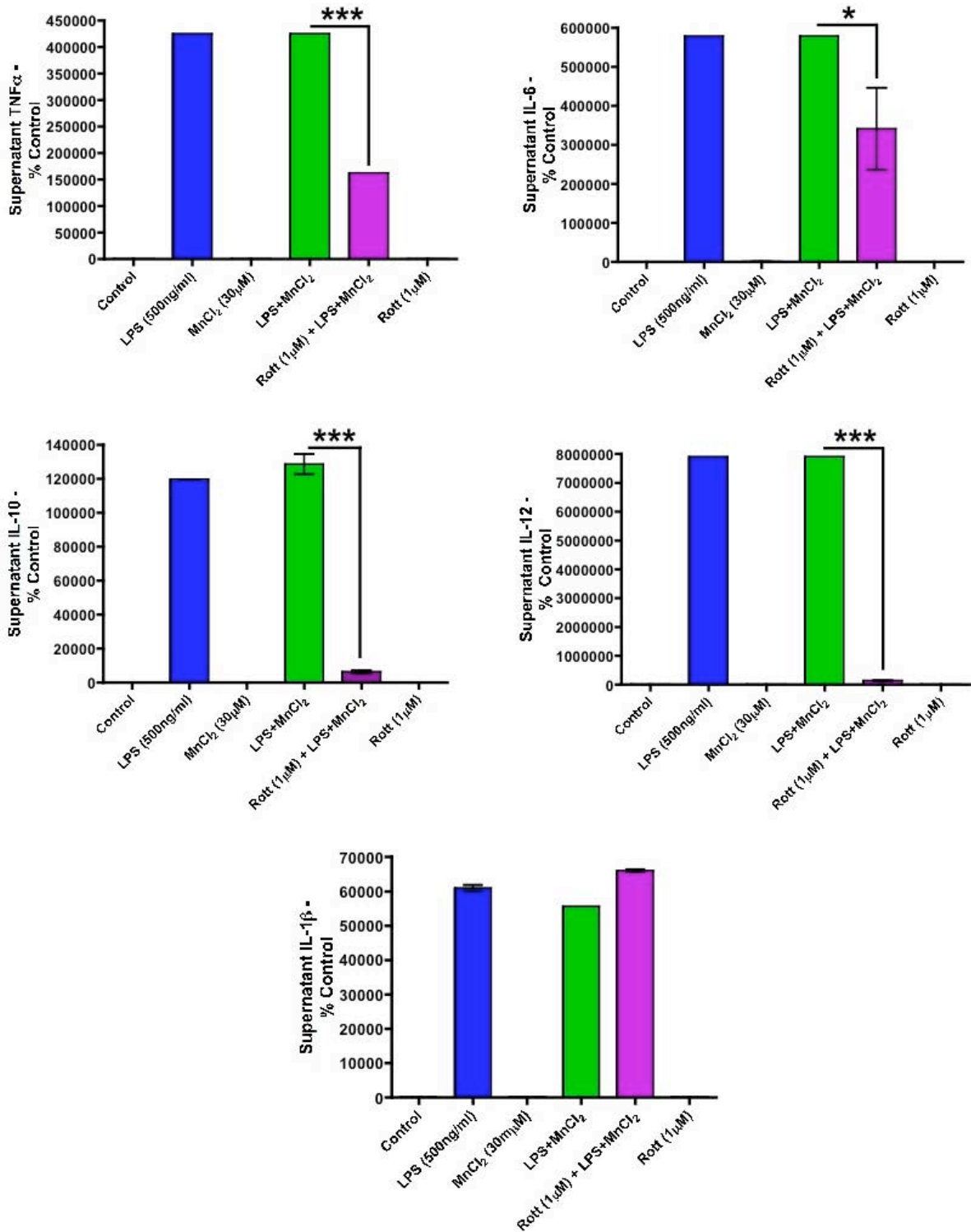


Figure 6. Primary microglia cytokine release.

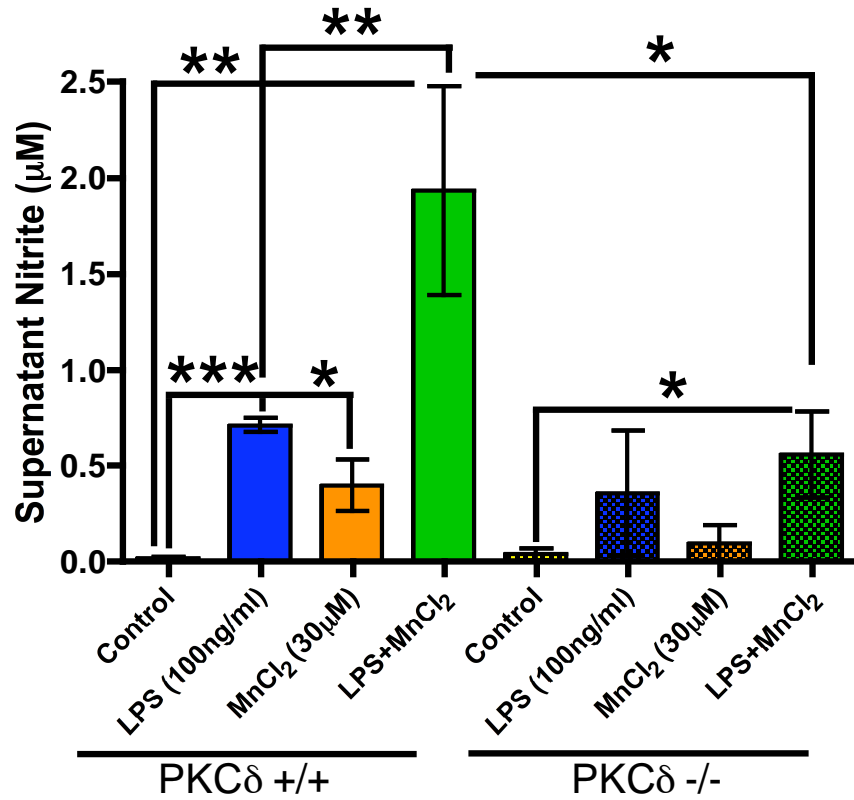


Figure 7. Supernatant nitrite concentrations from microglia cultured from wild-type and PKC δ knockout mice.

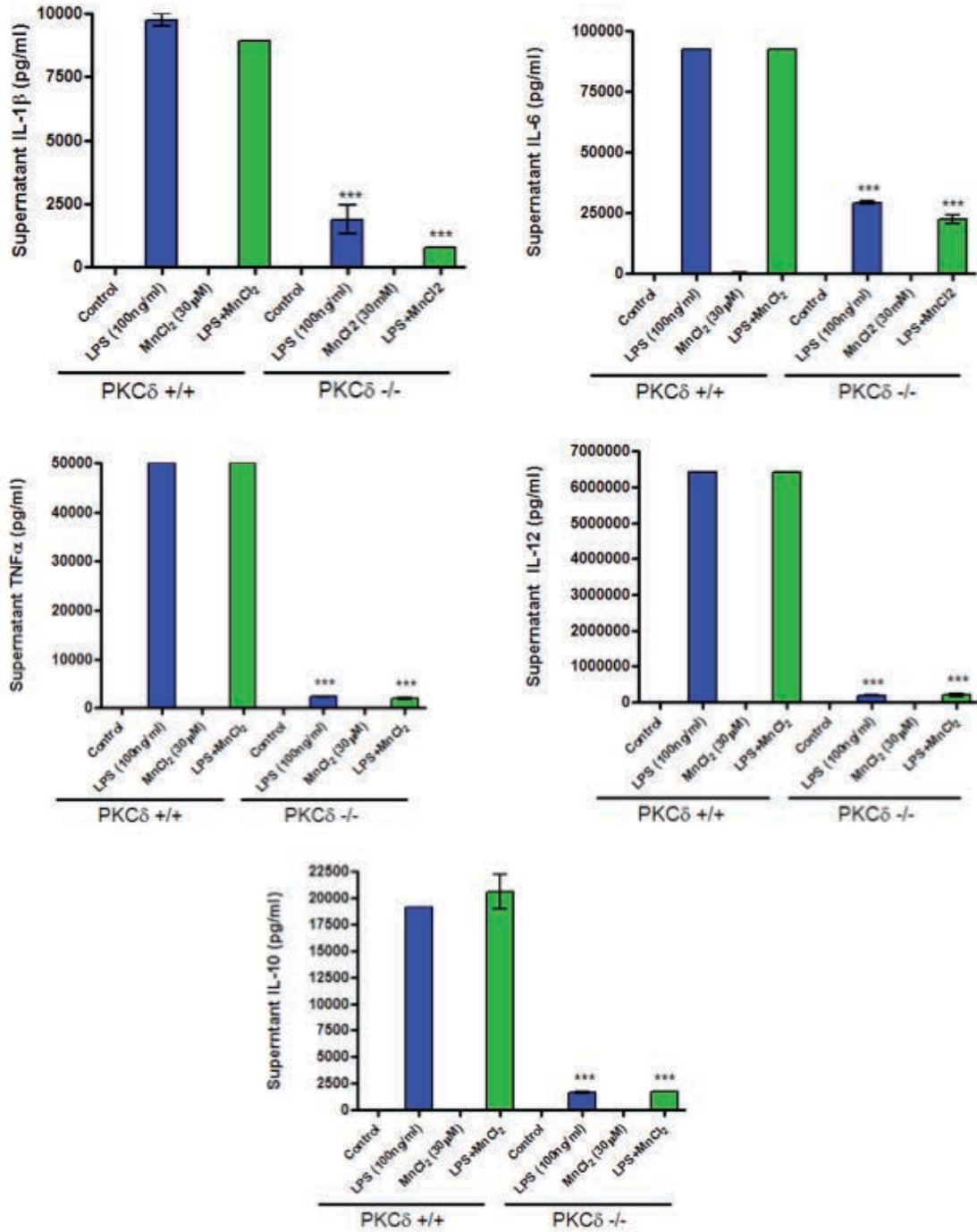


Figure 8. Cytokine release from microglia cultured from wild-type and PKC δ knockout animals.

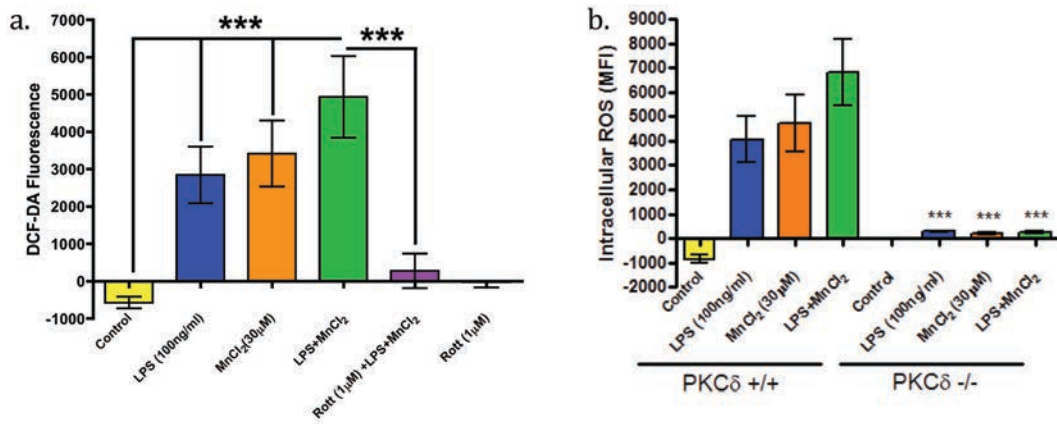


Figure 9. Intracellular ROS production was determined in a. primary microglia and b. primary microglia culture from wild-type and PKC δ knockout animals.

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CHAPTER III: CHARACTERIZATION OF THE ANTI-INFLAMMATORY FUNCTION OF DIAPOCYNIN IN CELL CULTURE LPS-INDUCED NEUROINFLAMMATION

Abstract

Neuroinflammation has gained increased recognition as a critical factor in neurodegeneration, specifically dopaminergic degeneration, and thus a contributing factor to the progression of Parkinson's disease (PD). Activation of microglia can instigate neuroinflammatory events leading to neurotoxicity through various molecular signaling pathways. In our study, we investigate the ability of diapocynin, an apocynin metabolite, to block the neuroinflammatory response caused by lipopolysaccharide (LPS), a known inflammatory stimulant. Using BV2 microglia cells (an immortalized mouse microglia cell line) and primary microglia we characterized the anti-inflammatory effects of diapocynin against LPS-induced neuroinflammation. We compare the efficacy of diapocynin to apocynin in each compounds ability to block specific neuroinflammatory events. First, the EC50 of each compound was determined by blocking LPS-induced iNOS activity. We found that the EC50 of diapocynin was about eight times less than that of apocynin. From this data, we established the concentrations, for each compound, to be used for the rest of the studies. We focused on key neuroinflammatory events: release of a variety of cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS).

First we show that diapocynin, at a concentration of 10 μM , is as effective blocking LPS-induced iNOS activation as apocynin, at a concentration of 100 μM . Diapocynin blocked the release of key inflammatory cytokines: IL-1 β , IL-6, IL-10, IL-12, and TNF α . Next we looked at ROS production by looking at intracellular ROS (iROS) and the expression of a protein subunit of the NADPH oxidase complex. We found that diapocynin was able to decrease gp91phox protein expression more effectively than apocynin in LPS stimulated BV2 microglia cells. Together, all the gathered data and results suggest that diapocynin has the potential to be a therapeutic agent against neuroinflammation.

Introduction

Neuroinflammation has gained increasing recognition as a critical factor in neurodegeneration, specifically dopaminergic (DA) neuronal degeneration, and thus a contributing factor to the progression of Parkinson's disease (PD). Apocynin, a naturally occurring methoxy-substituted catechol, has been used experimentally as a NADPH oxidase inhibitor (Simons, Hart et al. 1990; Stefanska and Pawliczak 2008). By inhibiting NADPH oxidase assembly, apocynin can decrease the production of superoxide from microglia. DA neurons are extremely sensitive to oxidative stress (Jenner and Olanow 1998), showing that blocking production of ROS is a pertinent therapy in the prevention of neurodegeneration. Though the mechanism has not yet been completely elucidated, it is believed that apocynin impairs the translocation of p47phox, a cytosolic component of NADPH oxidase, to the cell membrane (Peters,

Hiltermann et al. 2001; Barbieri, Cavalca et al. 2004). Tang's group has demonstrated the ability of apocynin to protect neurological function in a mouse model of reperfusion (Tang, Cairns et al. 2008). Apocynin also reduced infarct volume and the incidence of cerebral hemorrhage. These studies illustrate some of apocynin's therapeutic potential for neuroinflammatory diseases.

It has been determined that apocynin is a prodrug. Through peroxidase-mediated oxidation, apocynin is converted into a dimer (Stefanska and Pawliczak 2008). The apocynin dimer was observed, and shown to be more efficient than its parent compound apocynin (Johnson, Schillinger et al. 2002). In our study, we investigate the ability of diapocynin (5,5'-dehydrodiacetovanillone), an apocynin dimer, to block neuroinflammatory responses brought about in response to lipopolysaccharide (LPS) treatment. Our laboratory synthesized diapocynin through oxidative coupling of apocynin.

Using the active metabolite, diapocynin, we were able to block the release of key proinflammatory cytokines: IL-1 β , IL-10, IL-12, and TNF α in primary microglia and nitrite release from LPS stimulated BV2 microglia cells and primary microglia cells. We also found diapocynin can block the expression of the protein gp91phox, a subunit of NADPH-oxidase, in LPS stimulated BV2 microglia cells. Together, the results suggest that diapocynin has the potential to be a therapeutic agent against neuroinflammation.

Materials and Methods

See Chapter II.

Changes:

Manganese and rottlerin were not used in these studies.

Diapocynin and apocynin were used as 30-minute pretreatments.

Diapocynin was used at a concentration of 10 μM .

Apocynin was used at a concentration of 100 μM .

Results

Diapocynin is a more effective inhibitor than apocynin

In order to determine working concentrations for diapocynin and apocynin, as well as to directly compare the efficacy's of each compound we created EC50 curves of each compound in terms of blocking LPS-induced iNOS activation. Using BV2 cells, we induced nitrite production using LPS. We found the EC50 of diapocynin to 6.8 μM , almost eight times lower than apocynin's EC50, 54.4 μM (Figure 1). From this, we determined working concentrations for diapocynin and apocynin of 10 μM and 100 μM , respectively.

Diapocynin inhibited LPS-induced iNOS activation more effectively than apocynin

Using BV2 cells (Figure 2a) and primary microglia (Figure 2b), we assayed for iNOS activity indirectly through measurement of supernatant nitrite concentration. Both apocynin and diapocynin were used as 30-minute pretreatments, and the cells were then treated with LPS for 24-hours. We observe close to a 1200% increase in iNOS

activation upon LPS treatment, which is significantly attenuated with both diapocynin and apocynin pretreatment. The data also shows diapocynin is 10 times as effective as apocynin at blocking LPS-induced iNOS activation, as it was used at a concentration 10 times less than that of apocynin and both showed a similar iNOS activation inhibition.

Diapocynin effectively attenuates LPS-induced iROS production

Primary microglia were treated for 24-hours with LPS and diapocynin as a 30-minute pretreatment (Figure 3). LPS efficiently caused the cells to increase ROS production and sustain this production over 24 hours. Pretreatment with diapocynin effectively blocked all iROS production, bringing iROS concentration below basal levels.

Diapocynin blocked the LPS-induced release of specific cytokines as effectively as apocynin, in primary microglia

Using LPS we were able to elicit an increase in cytokine release from primary microglia. LPS treatment was allowed to run for 24 hours, while diapocynin and apocynin were both used as 30-minute pretreatments (Figure 4). Both diapocynin and apocynin were able to inhibit LPS-induced cytokine release of four or the five cytokines assayed for: IL-1 β , IL-10, IL-12, and TNF α . Neither compound was able to block the release of IL-6. Again, we observe the ability of diapocynin to block cytokine release as well as apocynin, at a lower concentration.

Diapocynin is able to decrease gp91phox protein expression

Gp91phox is the one of the two NADPH oxidase membrane bound subunits. The NADPH oxidase complex is one of the main contributors to ROS production, which is extremely deleterious in neural environments. Using Western blotting, we see 24-hour

LPS treatment causes a significant increase in gp91phox protein expression. This increased protein expression can be attenuated upon diapocynin pretreatment (30-minute) (Figure 5). However, apocynin is unable to block LPS-induced increases in gp91phox expression at a concentration of 100 μM compared to diapocynin's 10 μM working concentration.

Discussion

In this study we characterize the ability of the novel compound diapocynin to inhibit proinflammatory events that have been linked to progressive neurodegenerative diseases. Apocynin, a known NADPH oxidase inhibitor, is the parent compound of diapocynin, and was used in some of the assays as a comparison compound. We found the EC₅₀ of diapocynin to be approximately eight times less than the EC₅₀ of apocynin, in terms of blocking LPS-induced iNOS activity, 6.80 μM versus 54.37 μM respectively. For the remainder of the experiments we used diapocynin at a concentration of 10 μM and apocynin at a concentration of 100 μM . We observed the ability of diapocynin to inhibit LPS-induced iNOS activity, cytokine release, and gp91phox protein expression as well as or more effectively than apocynin, even with a working concentration 10 times less.

Recent research has shown that sustained inflammation may play a pivotal role in the development and progression of various neuroinflammatory diseases, including Parkinson's disease, Alzheimer's disease, and Huntington's disease (Streit, Mrazek et al. 2004; Whitton 2007). Microglia, one of the main contributors to neuroinflammation,

are part of the brain's innate immune system (Streit and Kincaid-Colton 1995; Whitton 2007). Activation of microglia occurs in response to a variety of triggers including pathogens, infection, and trauma, leading to a normal immune response. This inflammatory response is normally transient, providing inflammatory mechanisms that help to prevent injury while promoting healing. When these inflammatory mechanisms become chronically activated, and neuronal control over microglia is lost due to neuronal damage, the microglia reach a hyperactive state. At this point, it is believed that persistent neuroinflammation is reached, exacerbating present neuropathologies (Polazzi and Contestabile 2002). This neuroinflammatory response is characterized by the release of various factors including cytokines and chemokines, ROS, and RNS (Liu, Gao et al. 2003; Block and Hong 2007), many of which are produced by microglia.

All of the proinflammatory factors released during the neuroinflammatory response can be cytotoxic in nature, causing a toxic neuronal microenvironment. Though in particular, oxidative stress is extremely deleterious to dopaminergic (DA) neurons, as they are very sensitive to it (Jenner and Olanow 1998), but DA neurons also tend to produce abundant quantities of ROS (Whitton 2007) due to dopamine content. Oxidative stress is reached when endogenous antioxidant systems are saturated and can no longer handle the amount of ROS being generated. The brain appears to be extremely susceptible to oxidative stress owing to its excessive oxygen demands (Halliwell 2001). Under normal physiological conditions, low levels of ROS are generated, but are scavenged by endogenous antioxidants including superoxide dismutase, glutathione peroxidase, and catalase as well as by small molecules like

vitamin C and E (Halliwell 2001; Halliwell 2006). ROS can wreak havoc on a variety of macromolecules, irreversibly modifying them through oxidation. Proteins, carbohydrates, lipids, and DNA are all at risk of ROS mediated modification. All of these points make antioxidants extremely appealing therapies for neurodegenerative diseases.

Nitrosative stress can also give rise to a cytotoxic environment. Nitric oxide (NO) is an important signaling molecule within the brain, and is necessary for normal brain function. Increases in both ROS and NO can lead to the generation of a secondary oxidizing specie peroxynitrite, as well as peroxynitrous acid. Peroxynitrite has the ability to cause oxidative damage in numerous proteins namely tyrosine hydroxylase (an enzyme necessary for dopamine production) and α -synuclein (Ara, Przedborski et al. 1998; Przedborski, Chen et al. 2001; Hirsch and Hunot 2009). Increases in production of ROS can also lead to a loss of NO bioavailability, which can disrupt pathways that use NO as a signaling molecule. We show diapocynin is able to attenuate both nitrite release and intracellular ROS production (Fig.2, Fig.3).

In Figure 4, we show diapocynin's ability to block the release of all cytokines assayed for excluding IL-6. IL-6 is classically thought of as a proinflammatory cytokine, though studies have shown that IL-6 actually has anti-inflammatory as well as regenerative activities (Tilg, Trehu et al. 1994; Opal and DePalo 2000; Scheller, Chalaris et al. 2011). It has also been shown that IL-6 down-regulates the synthesis of IL-1 and TNF α , while having little effect on IL-10, which is technically the functional definition of an anti-inflammatory cytokine (Libert, Takahashi et al. 1994; Xing, Gauldie et al. 1998).

Though IL-6 is still considered a marker for systemic activation of proinflammatory cytokines (Barton 1997), playing a pivotal role in acute-phase protein response, it also plays a role in modulating the immune response through both pro- and anti-inflammatory events (Barton, Shortall et al. 1996).

In conclusion, these data demonstrate the ability of diapocynin, a metabolite of apocynin, to efficiently block LPS-induced neuroinflammation in a cell culture model. Our study first compares the EC50 of diapocynin to apocynin, in their ability to block LPS-induced iNOS activity, and find diapocynin to be an almost eight times more potent inhibitor. Using working concentrations of 10 μM for diapocynin and 100 μM for apocynin, we find diapocynin is just as effective at inhibiting the release of IL-1 β , IL-10, IL-12, and TNF α , as well as the LPS-induced increases in gp91phox protein expression. Diapocynin also efficiently attenuated the increase in iROS production seen upon LPS treatment. Collectively, these results indicate that diapocynin is a more efficient anti-inflammatory than apocynin, and may have potential therapeutic usefulness in neuroinflammatory disorders.

Acknowledgements

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Figure Legend

Figure 1. Diapocynin is a more effective inhibitor of iNOS activity. LPS was used to induce iNOS activity in BV2 cells in order to create dose-response curves and determine an EC50 for each apocynin (apo) and diapocynin (diapo). LPS was used at a concentration of 1 µg/ml, and various doses of apocynin (Fig. 1b) and diapocynin (Fig. 1a) (n=8). iNOS activity was indirectly measured via supernatant nitrite concentration. An EC50 of 54.37 µM and 6.80 µM was obtained for apocynin and diapocynin, respectively.

Figure 2. Diapocynin can inhibit nitrite release more efficiently than apocynin. LPS-induced iNOS activation was attenuated using both diapocynin (diapo) and apocynin (apo) in both BV2 cells (n=17, diapo and apo n=8) and primary microglia (n=7). LPS was used as a 24-hour treatment, both diapocynin and apocynin were used as 30-minute pretreatments. Diapocynin was used at a concentration of 10 µM and apocynin at a concentration of 100 µM. We observed that both compounds were able to significantly reduce LPS-induced iNOS activity. These data indicate that diapocynin is more effective at blocking this increase in iNOS activity than apocynin. Neither diapo nor apo effected iNOS activation when used alone (***) denotes a p-value < 0.001 as determined by one-way ANOVA).

Figure 3. Diapocynin is able to inhibit production of intracellular ROS (iROS). The fluorescent probe DCF-DA was used to assay for iROS. Primary microglia were treated

with LPS for 24-hours and diapocynin (diapo) was used as a 30-minute pretreatment (n=6). We observe LPS can increase MFI from essentially 0 to over 6000 and the ability of diapocynin to bring it back down to almost below basal level. Diapo used alone had no effect on iROS production. (***) denotes a p-value < 0.001, as determined by one-way ANOVA).

Figure 4. Diapocynin was able to block LPS-induced cytokine release of 4 or the 5 cytokines quantified. Cells treated with LPS for 24-hours, using diapocynin (diapo) and apocynin (apo) as a 30-minute pretreatment (n=4). Five cytokines were assayed for: IL-1 β , IL-6, IL-10, IL-12, and TNF α . LPS efficiently increased the release of all five cytokines. Both diapo and apo significantly inhibited the release of four of the five cytokines, neither was able to attenuate the release of IL-6. (***) denotes p-value < 0.001, as determined by one-way ANOVA).

Figure 5. Diapocynin is able to attenuate LPS-induced increases in gp91phox protein expression. Western blotting was used to quantify the expression of the protein gp91phox. BV2 cells were seeded into T-75 flasks and treated with LPS for 24-hours, using apocynin (apo) and diapocynin (diapo) as 30-minute pretreatments (n=3). Using densitometric analysis, we normalize the bands to the β -actin band. LPS is able to significantly increase the expression of gp91phox. Diapo significantly blocked LPS-induced increase in gp91phox protein expression while apo did not have much of an effect. Again we see the ability of diapo to attenuate a proinflammatory event more

effectively than apo when used at a concentration 10 times less. (* denotes p-value < 0.05, as determined using unpaired t-test).

Figures

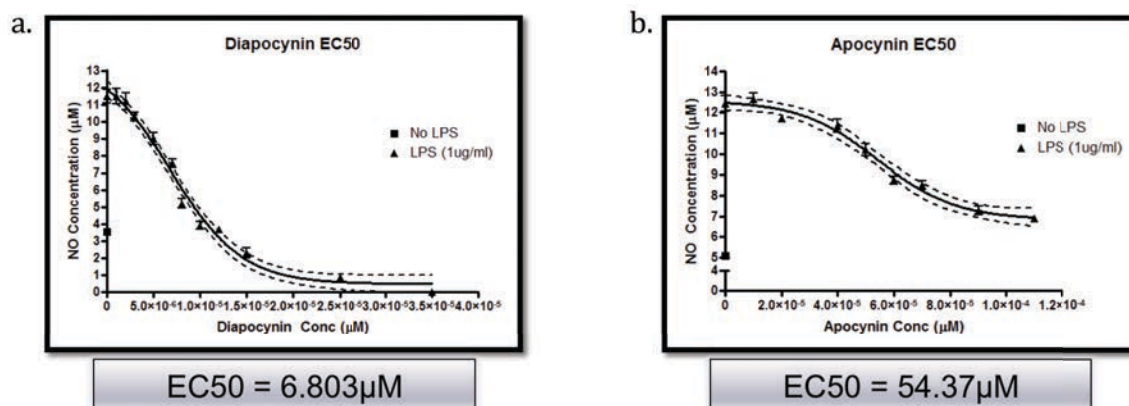


Figure 1. EC50 curves for diapocynin and apocynin using LPS-induced iNOS activity.

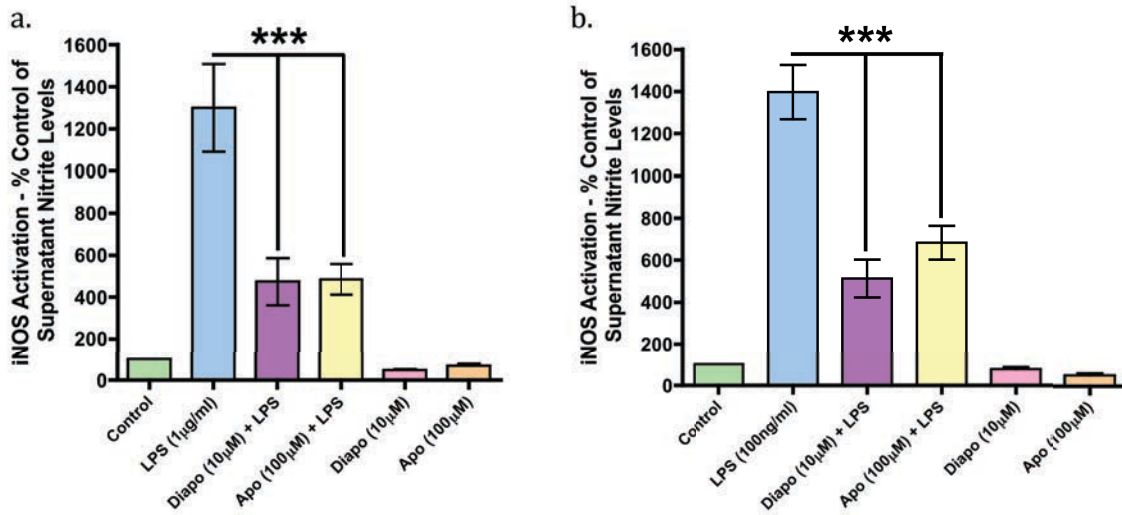


Figure 2. LPS-induced iNOS activity attenuation by diapocynin and apocynin.

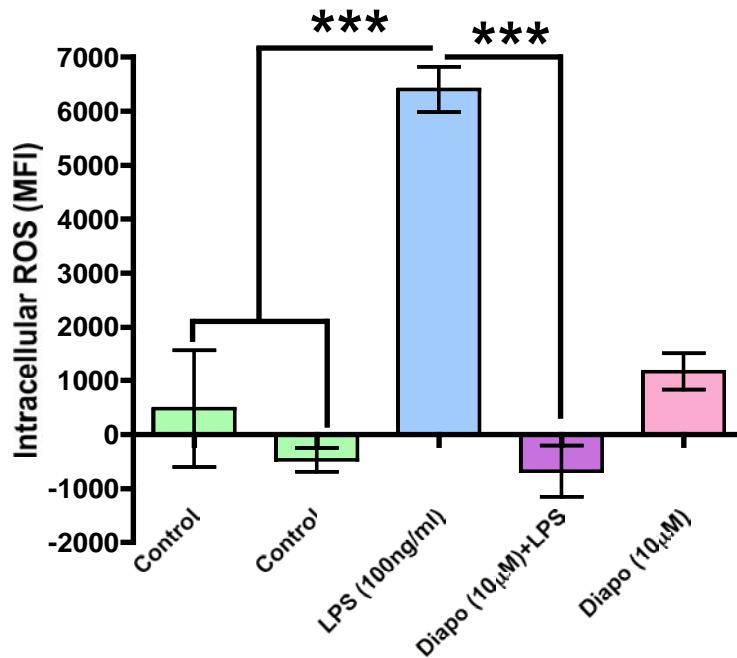


Figure 3. LPS-induced iROS production in primary microglia, which was inhibited by diapocynin pretreatment.

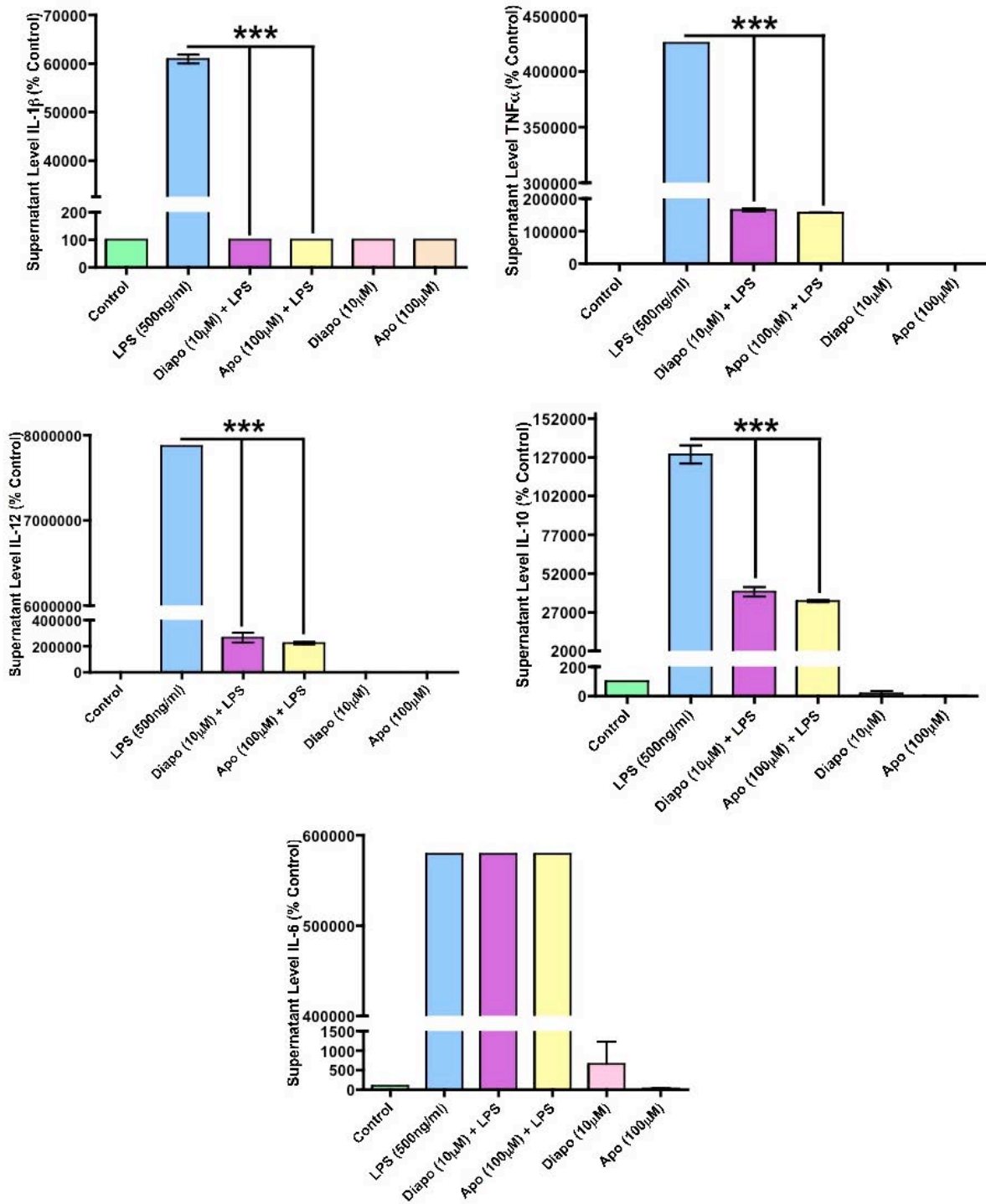


Figure 4. Cytokine release from primary microglia was increased using LPS. Both diapocynin and apocynin were able inhibit this release in four of the five cytokines.

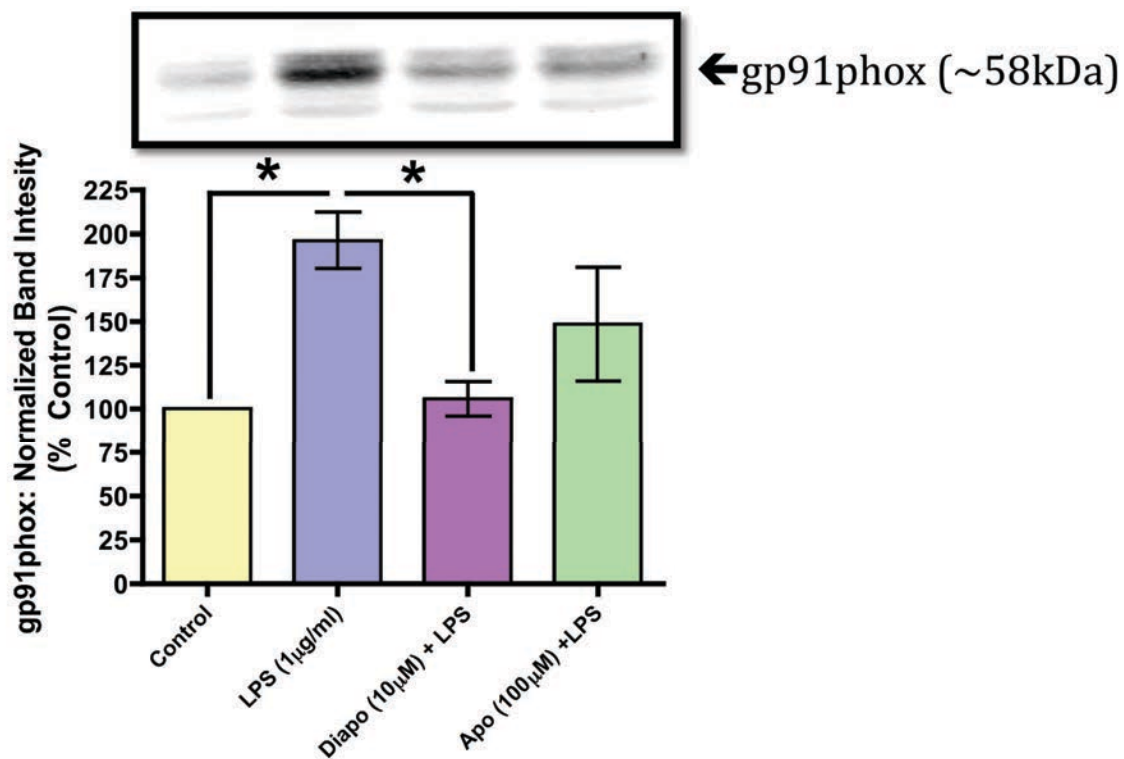


Figure 5. Western blot analysis showed what appeared to be an increase in expression upon LPS treatment, which was attenuated with diapocynin, but not apocynin.

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**CHAPTER IV: NOVEL NADPH OXIDASE MODULATOR PROTECTS
AGAINST NEUROINFLAMMATION ASSOCIATED WITH LPS/MANGANESE
NEUROTOXICITY**

Abstract

Sustained neuroinflammation has become increasingly evident as a causative factor in the progression of many neurodegenerative diseases including Parkinson's disease (PD). Exposure to environmental neurotoxicants such as metals and pesticides are considered potential risk factors in the pathogenesis of PD. Microglia are an integral part of the innate immune system of the CNS and are capable of producing rapid neuroinflammatory responses when exposed to pathogens or neurotoxic insults. Metal exposure has been shown to trigger activation of both microglia and astroglia in order to counter neurotoxic insults. Recently, studies have shown that manganese (Mn) exposure by itself or in combination with other inflammatory stimuli can produce neuroinflammatory responses in the nigrostriatal dopaminergic system. We, and others, have shown that Mn can augment lipopolysaccharide (LPS)-induced cytokine production, iNOS activation, and ROS generation in microglial cells. In the present study, we sought to investigate the anti-inflammatory potential of a novel NADPH oxidase inhibitor diapocynin against microglial activation in response to Mn/LPS-induced inflammatory events. Diapocynin treatment significantly blocked Mn/LPS-induced iNOS activation and ROS generation in both primary microglia and BV2 microglia cell models. Measurement of cytokine release in the supernatant of

diapocynin treated BV2 cell lines and primary microglia cells revealed that the compound effectively suppressed the release of TNF α , IL-1 β , IL-6, IL-10 and IL-12. Expression of phox proteins, components of the NADPH oxidase complex and a main contributor to ROS production, was also decreased with diapocynin treatment. Collectively, these results suggest that our translational approach with diapocynin can suppress neuroinflammatory events associated with neurotoxic metal exposure.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, but the etiology is currently unknown (Nussbaum and Ellis 2003). The disease is mainly characterized by the progressive degeneration of nigral dopaminergic neurons and loss of striatal dopamine. Neuroinflammation has gained increasing recognition as a critical factor in the progression of neurodegenerative processes underlying PD. Activation of microglia can instigate neuroinflammation leading to neurotoxicity through various molecular signaling pathways. Throughout the past decade, compelling studies have shown these neuroinflammatory events to be self-perpetuating pathogenic processes (Block, Zecca et al. 2007). Therefore, it stands to reason that an anti-inflammatory compound could be useful in the treatment of PD. Microglia, one of the resident immune cell types in the brain, play a key role in microenvironment surveillance and injury repair, mainly through the activation of inducible NADPH oxidase (NOX2) (Zhang, Wong et al. 2009). Therefore, NOX-2

inhibitors may be useful in the treatment of PD through inhibiting sustained neuroinflammatory events that can lead to dopaminergic neuronal degeneration.

Exposure to transition metals such as manganese (Mn) and iron has been linked to pathogenesis of PD. Notably, recent studies show Mn treatment can not only induce neuroinflammatory responses in *in vitro* and *in vivo* models, but also augment these responses (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006; Liu, Sullivan et al. 2006; Moreno, Sullivan et al. 2008). We, and others, have shown that Mn can augment lipopolysaccharide (LPS)-induced cytokine production, NOX-2 activation, and ROS generation in microglial cells.

The plant phenol, apocynin, a naturally occurring methoxy-substituted catechol has been used by various research groups as a NADPH oxidase inhibitor (Simons, Hart et al. 1990; Stefanska and Pawliczak 2008). The NOX2 NADPH oxidase complex, which is the focus of this paper, is made up of five phox proteins as well as the GTPase Rac. The membrane bound subunits are gp91phox and p22phox. Gp91phox is believed to have six transmembrane domains comprised of various cytosolic factors, and is considered to be the organizing subunit. The exact role of p22phox is unknown, but it is known that flavocytochrome *b₅₅₈* is a heterodimer consisting of gp91phox and p22phox (Parkos, Allen et al. 1987). The other three phox proteins are cytosolic and translocate to the membrane. The subunit p47phox is believed to organize the translocation of itself and p67phox, p40phox, and GTPase Rac to the membrane, where they connect with the membrane bound portion of the complex. All of these cytosolic proteins fail to translocate to the membrane in the absence of p47phox (Heyworth, Curnutte et al.

1991; Dusi, Donini et al. 1996). The activator subunit is believed to be p67phox, which needs to be directly in contact with gp91phox, activating the complex through protein-protein interactions (Clark, Volpp et al. 1990; Nauseef 2001). The final cytosolic phox protein, p40phox, is actually dispensable, and not necessary for NOX2 activity, though it does seem to enhance oxidase function (Cross 2000; Kuribayashi, Nuno et al. 2002; Bedard and Krause 2007). Apocynin is believed to impair the translocation of p47phox to the cell membrane, thus keeping NOX2 from becoming activated, though the complete mechanism has not yet been elucidated (Peters, Hiltermann et al. 2001; Barbieri, Cavalca et al. 2004). We hypothesize that diapocynin works in a similar fashion.

Apocynin has been shown to convert into a dimer through peroxidase-mediated oxidation (Stefanska and Pawliczak 2008), which was shown to be more efficient than its parent compound (Johnson, Schillinger et al. 2002). Our lab synthesized an apocynin dimer, diapocynin (5, 5'-dehydrodiacetovanillone), and previously found it to be more effective than apocynin at blocking lipopolysaccharide (LPS)-induced inflammatory responses in microglia cell culture models. Herein, we determine the efficacy of the newly synthesized novel compound diapocynin in attenuating the LPS/Mn-induced inflammatory response. We hypothesized that diapocynin can dampen neuroinflammatory events induced by cotreatment of LPS and manganese in cell culture models of brain neuroinflammation.

Materials and Methods

See Chapter II.

Changes:

Rottlerin was not used in this study.

Diapocynin was used at a concentration of 10 μ M.

Immunocytochemistry. Magnetically separated microglia were plated at 150,000 cells per well into poly-D-lysine coated 24-well plates containing coverslips. The cells were allowed to attach overnight and were then treated with 100 ng/ml LPS, 30 μ M Mn, or a combination. Diapocynin was used as a 30-minute pretreatment in one group at a concentration of 10 μ M. Upon treatment end the cells were fixed in 4% PFA for 30 minutes. Cells were then blocked in buffer containing 2% BSA (Sigma-Aldrich, St. Louis, MO), 0.01% Triton-X (Sigma-Aldrich, St. Louis, MO), and 0.01% Tween-20 (Bio-Rad, Hercules, CA). The primary antibody used was p67phox (1:100) (rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and was incubated on the cells overnight. Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:1000) (Invitrogen, Carlsbad, CA) was used as secondary for 1 hour. Cells were incubated in Hoechst (Invitrogen, Carlsbad, CA) for 5 minutes before cover slips were mounted on slides using Fluoromount (Sigma, St. Louis, MO).

Results

Diapocynin attenuates LPS/Mn-induced iNOS activation in BV2 cells

Previous studies have outlined the ability of Mn to potentiate LPS-induced inflammatory events, such as nitrite production (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006). Here, we determined diapocynin can be used as an inhibitory compound, blocking LPS/Mn-induced proinflammatory events (Figure 1). First, we observed the ability of Mn to potentiate LPS-induced iNOS activation. Diapocynin, used as a 30-minute pretreatment, greatly attenuates LPS/Mn-induced increases in iNOS activity.

Diapocynin inhibits nitrite production and iNOS activation in primary microglia

We used primary microglia to repeat the iNOS activation study, as primary microglia are a more accurate model. The cells were treated with LPS and Mn for 24-hours, using diapocynin as a 30-minute pretreatment (Figure 2). Again we observe Mn potentiation of LPS in terms of iNOS activation and nitrite production. Mn treatment alone did not stimulate an increase in nitrite production, while it is able to significantly increase LPS-induced nitrite production, as compared to LPS treatment alone. Diapocynin is able to significantly inhibit nitrite release, correlating to a decrease in iNOS activation, as nitric oxide is a main product of iNOS activity.

Diapocynin modulates LPS/Mn-induced increase in cytokine release in BV2 cells

Using BV2 cells, we probed for the release of specific cytokines using the Luminex bead-based immunoassay. LPS and Mn were used as 24-hour treatments and diapocynin as a 30-minute pretreatment. The supernatant was collected and probed for IL-6, IL-12, and TNF α (Figure 3). IL-6 alone shows statistically significant trends, both

in terms of Mn potentiation and inhibition of cytokine release through diapocynin pretreatment. These trends are seen in IL-12 and TNF α when comparing means, but are were not found significant upon statistical analysis.

Increased cytokine release in primary microglia upon LPS/Mn treatment is inhibited by diapocynin

Primary microglia were used to repeat cytokine release analyses to better understand what might happen *in vivo*. Diapocynin was first used as a 30-minute pretreatment, then cells were treated in LPS and Mn for 24-hours before the supernatant was collected. Five cytokines were probed for in the collected supernatant: IL-1 β , IL-6, IL-10, IL-12, and TNF α (Figure 4). The release of IL-10 was not increased by Mn treatment, but LPS with Mn significantly increases the release of this cytokine in comparison to LPS treatment alone. Diapocynin was able to attenuate the LPS/Mn-induced cytokine release in all cytokines except IL-6.

Diapocynin attenuates intracellular ROS (iROS) production in primary microglia

Production and release of ROS are known to be exceedingly toxic to tissues in the surrounding area as it causes a cytotoxic environment and can exert deleterious effects directly upon a cell (Bedard and Krause 2007). As ROS production can occur very rapidly after an insult, we measured iROS at two time points to assess whether diapocynin could inhibit the production of early ROS as well as sustain its inhibitory effects over a long period. Using primary microglia, we treated with LPS and Mn for 6-hours and 24-hours, using diapocynin each time as a 30-minute pretreatment (Figure 5). Using the fluorescent probe DCF-DA, we quantified iROS as an MFI. We observe that

Mn is a more potent activator of iROS production than LPS. Instead of potentiation between LPS and Mn in terms of iROS production, we observe more of an additive relationship, especially when looking at the 24-hour treatment group. Diapocynin is able to inhibit iROS production at both 6 and 24 hours, showing it is able to work quickly to inhibit iROS production, as well as continue inhibition through sustained activity.

Diapocynin effects on gp91phox protein expression

Using Western blotting and BV2 cells, we analyzed the protein expression of gp91phox (Figure 6). Though the results were not statistically significant, we observe an increase in the mean gp91phox protein expression upon the 24-hour treatment of LPS and LPS/Mn treatment. Diapocynin, as a 30-minute pretreatment decreases the mean gp91phox protein expression near to the basal level mean, if not slightly below.

Microglia morphology greatly changes upon LPS and LPS/Mn treatment, but can be returned to a quiescent morphological state with diapocynin treatment

Primary microglia cells were stained with p67phox, one of the cytosolic subunits of the NADPH oxidase complex, and the nuclear stain Hoechst after 24-hour treatment with LPS and Mn and 30-minute pretreatment with diapocynin (Figure 7). We see morphology consistent with a 'resting' state in the control group. Microglia in a 'resting' state have more of a ramified morphology, with distinct branches reaching from the cell body into the environment. In the LPS group and LPS/Mn groups, we observe a more amoeboid-like shape, consistent with activated microglia morphology. The protein p67phox follows a honeycomb-like pattern within the cell, with, what appears to be,

increased density at the cell membrane, which is expected as p67phox translocates from the cytosol to the membrane in order to activate gp91phox. Treatment with Mn alone does not appear to have much effect on morphology, and the cells remain in their ramified state. Pretreating the cells with diapocynin appears to not only attenuate p67phox expression, but also keeps the microglia from becoming activated, as they are morphologically similar to the control group cells, as compared to the LPS and LPS/Mn groups' amoeboid appearance.

Discussion

In this study we observe the ability of novel compound diapocynin to inhibit proinflammatory events associated with LPS/Mn insult. These same proinflammatory events that are elicited upon insult with LPS/Mn have been linked to neuroinflammation and are believed to exacerbate underlying neuropathologies (Polazzi and Contestabile 2002). Using diapocynin at a concentration of 10 μ M, we observe its ability to inhibit iNOS activation, cytokine release, iROS production, and microglial morphological changes. We also observe trends in the expression of gp91phox, diapocynin showing what appears to be inhibition in gp91phox protein expression, which appears to be increased upon treatment with LPS and Mn together.

The CNS is able to self-regulate its innate immune system, making it an immune privileged system. It is necessary for peripheral immune cells to cross an intact blood brain barrier, regulation of macrophages and lymphocytes is actively accomplished by neurons and glia, and immunocompetent microglia are responsible for directing

lymphocytic neuroprotective responses (Carson, Doose et al. 2006; Harry and Kraft 2008). Astrocytes and microglia are the main glial cells of the brain's innate immune system, though microglia are believed to be the main neuroinflammatory contributors (Streit and Kincaid-Colton 1995; Whitton 2007). Microglia continually palpate their environment, becoming rapidly activated in reaction to any environmental change or trigger including pathogens, infection, and trauma. Microglial neuroinflammatory responses are characterized by the release of cytokines and chemokines, ROS, and RNS (Liu, Gao et al. 2003; Block and Hong 2007; Block, Zecca et al. 2007), all of which are observed in the present study. Microglia also undergo morphological changes upon activation, going from what is considered a 'resting' state, though it has been shown that microglia are continually active and constantly surveying their environment, characterized by a ramified morphology to an activated state characterized by an amoeboid-like morphology. We see both morphologies in Figure 7, where LPS and LPS+Mn are able to elicit an amoeboid-like morphology consistent with activated microglia, which is attenuated when cells are pretreated with diapocynin.

Manganese (Mn) is an essential trace element playing pivotal roles in various biological processes, functioning as an important cofactor for a variety of enzymes. These enzymes are critical in CNS homeostasis of both metabolic and redox reactions. The enzymes glycosyltransferase, pyruvate decarboxylase, glutamine synthetase, and superoxide depend upon manganese for their ability to properly function (Gonzalez-Zulueta, Ensz et al. 1998; Keen, Ensunsa et al. 2000; Takeda 2003). Using specific

carriers including transferrin and divalent metal transporter 1 and through simple diffusion, Mn is able to cross the blood brain barrier (Yokel and Crossgrove 2004).

Exposure to high amounts of Mn can lead to the development of a disorder known as manganism. People afflicted by this disorder display motor symptoms that very closely mimic symptoms associated with PD (Josephs, Ahlskog et al. 2005; Aschner, Guilarte et al. 2007). Due to these observations, researchers began looking into the mechanisms through which Mn works and if there may be a connection between Mn exposure and PD. Postmortem studies, using PD patient brains, have shown that though Mn-induced neuronal damage is prominent in the striatum and other structures of the basal ganglia, it does not show preferential targeting of the dopaminergic neurons (Perl and Olanow 2007). High dose Mn does, however, elicit an inflammatory response, increasing iNOS, ROS, proinflammatory prostaglandins, and other inflammatory mediators, and alterations which can lead to an excitatory neurotoxic response (Liu, Gao et al. 2002; Erikson and Aschner 2003; Erikson, Dorman et al. 2007). Being that Mn-induced neurotoxicity is associated with oxidative damage, free radical production, and neuroinflammation, which have all been implicated in the pathology of numerous neurodegenerative diseases, including PD, it appears to be an environmental factor that may play a role in development or exacerbation of neurodegenerative disorders.

Sustained neuroinflammation is believed to play a pivotal role in the development and subsequent progression of many neurodegenerative disorders, including PD (Streit, Mrazek et al. 2004; Whitton 2007), demonstrating that

neuroinflammation may be an important therapeutic target for PD, Alzheimer's disease, Huntington's disease, and many others. It is also believed that numerous factors: environmental factors, genetics, and cellular dysfunction, play a role in the etiology of neuroinflammatory disorders, known as a 'multiple hit hypothesis' (Carvey, Punati et al. 2006; Sulzer 2007), leading to a complex, multi-factorial etiology of neurodegeneration. In this study, we show how the compound diapocynin can inhibit various neuroinflammatory events associated with LPS/Mn insult, which could correspond to an increase in neuronal survival in the face of environmental neurotoxicant insult.

Dopaminergic neurons exhibit high sensitivity to oxidative stress, making ROS particularly toxic (Jenner and Olanow 1998). Pretreatment of cells with diapocynin inhibits iROS production, which was induced by LPS/Mn treatment (Figure 5). This inhibition was seen at an early time point of 6 hours, which was chosen as ROS production is seen early in microglial activation, and was sustained throughout a 24 hour period. It is also important to mention that iNOS activity was also attenuated by diapocynin pretreatment in both BV2 cells (Figure 1) and primary microglia (Figure 2). Superoxide anions and NO, though not extremely reactive by themselves, can react with one another to produce the highly reactive nitrogen species peroxynitrite, which has the ability to damage a variety of proteins through oxidation (Ara, Przedborski et al. 1998; Przedborski, Chen et al. 2001; Hirsch and Hunot 2009).

In conclusion, this study demonstrates the ability of the novel compound, diapocynin, to attenuate the neuroinflammatory response elicited by treatment with both LPS and Mn. We show that diapocynin has the ability to block LPS/Mn-induced

iNOS activity and the release of certain cytokines in a microglia cell line as well as in primary microglia. Diapocynin also inhibits LPS/Mn-induced production of iROS in primary microglia. We also observe gp91phox protein expression trends in BV2 cells, where diapocynin appears to inhibit the increase in protein expression that seems to be elicited by LPS/Mn treatment. Finally, we visualize the morphological changes that occur in microglia upon LPS/Mn treatment, which are seen to a lesser extent with diapocynin pretreatment, as well as p67phox expression changes. Collectively, these results indicate that diapocynin may have potential therapeutic usefulness in neuroinflammation and diseases associated with neuroinflammation.

Figure Legend

Figure 1. Diapocynin is able to attenuate LPS/Mn-induced nitrite production. BV2 cells were pretreated with diapocynin (diapo) for 30-minutes before being treated for 24-hours. The supernatant was collected and assayed for nitrite concentration (n=17, n=8 diapo grp), and the results were graphed as a percent of the control level. We observed Mn was able to potentiate the LPS-induced iNOS activity. Diapocynin pretreatment was able to significantly reduce LPS/Mn-induced iNOS activation. (***) denotes p-value < 0.001 as determined by one-way ANOVA).

Figure 2. Nitrite production was blocked by diapocynin. Primary microglia were used to show the ability of diapocynin (diapo) to attenuate LPS/Mn-induced iNOS activation. Primary microglia were seeded into 96-well plates at 150,000 cells per well and treated for 24-hours, with a 30-minute pretreatment of diapocynin. The supernatant was collected and probed for nitrite (n=9, n=8 diapo grp). Results were graphed as nitrite concentration. Mn potentiation was seen in iNOS activity, which diapocynin is able to attenuate. (** denotes p-value < 0.01, *** denotes p-value < 0.001, as determined by one-way ANOVA).

Figure 3. Diapocynin was able to modulate cytokine release in BV2 cells. BV2 cells were seeded into 96-well plates at 30,000 cells per well, pretreated with diapocynin (diapo) for 30-minutes, and then treated for 24-hours. Supernatant was collected and probed for IL-6, IL-12, and TNF α (n=4). It appears that LPS and Mn are able to increase the

release of each of the three cytokines, though only IL-6 shows statistically significant results. We also observe the ability of diapocynin to significantly reduce the amount of IL-6 and IL-12 being released, the same trend appears with TNF α , though no significance was found. (* denotes p-value < 0.05, *** denotes p-value < 0.001 as determined by one-way ANOVA).

Figure 4. Diapocynin attenuate LPS/Mn-induced release of various cytokines. Primary microglia were seeded into 96-well plates at 100,000 cells per well. Cells were pretreated for 30-minute with diapocynin (diapo) and then treated for 24-hours (n=4). The collected supernatant was probed for IL-1 β , IL-6, IL-10, IL-12, and TNF α . Here we observe the ability of the LPS+Mn cotreatment to greatly increase the release of each cytokine. Diapocynin is able to attenuate this increase in cytokine release in four of the five cytokines, IL-6 release is unaffected by diapocynin pretreatment. We also see Mn potentiation of LPS-induced cytokine release in IL-10. (***) denotes p-value < 0.001 as determined by one-way ANOVA).

Figure 5. Diapocynin blocked LPS/Mn-induced intracellular ROS production. Using primary microglia plate at 50,000 cells per well in 96-well plates, we probed for intracellular ROS (iROS) production at two time points. The fluorescent probe DCF-DA was used to assay for iROS, which was quantified as MFI. Cells were pretreated for 30-minutes before being treated for 6-hours and 24-hours (n=4). Here we observe the

ability of diapocynin to inhibit LPS/Mn-induced iROS production at both 6 hours and 24 hours. (***) denotes p-value < 0.001 as determined by one-way ANOVA).

Figure 6. Trends in the expression of gp91phox show modulation of LPS/Mn-induced increase in protein expression by diapocynin. Western blotting was used to observe any changes in the protein expression of gp91phox. BV2 cells were treated in T-75 flasks for 24-hours, using diapocynin as a 30-minute pretreatment (n=3). Densitometric analysis was done, normalizing all bands to the β -actin band. Due to cell variability, we were only able to see a significant increase in gp91phox protein production upon LPS treatment. It appears that the cotreatment of LPS and Mn was also able to increase protein expression when comparing the means, though no significance was found. Diapocynin decreases the mean LPS/Mn-induced increases in gp91phox expression, though no significance was found. (** denotes p-value < 0.01 as determined by unpaired t-test).

Figure 7. Morphological changes in microglia were visualized. A decrease in activated morphology was seen with diapocynin treatment. Immunocytochemistry was used to show microglial morphological changes, as well as p67phox expression. Cells were plated at 150,000 cells per well in 24-well plates, pretreatment with diapocynin (diapo) was 30-minutes with LPS and Mn treatments of 24 hours. We observed the normal ramified morphology of 'resting' microglia in the control group, as well as in the Mn only treatment group, which indicates the low dose of Mn is not activating them. Upon

LPS or LPS+Mn treatment, we observe drastic morphological changes as the cells go from their 'resting' morphology to an amoeboid like morphology, indicating the microglia have become activated. The cells pretreated with diapocynin show morphology more consistent with 'resting' microglia, though they do have a slight amount of rounding indicating there may be some activation occurring.

Figures

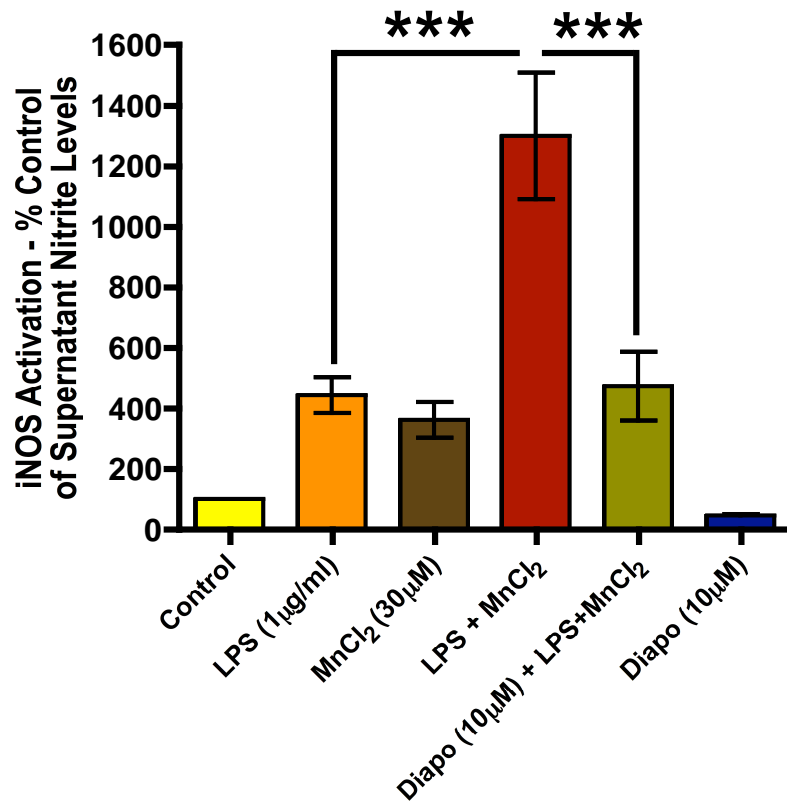


Figure 1. LPS/Mn-induced iNOS activity can be attenuated by diapocynin and apocynin in BV2 cells.

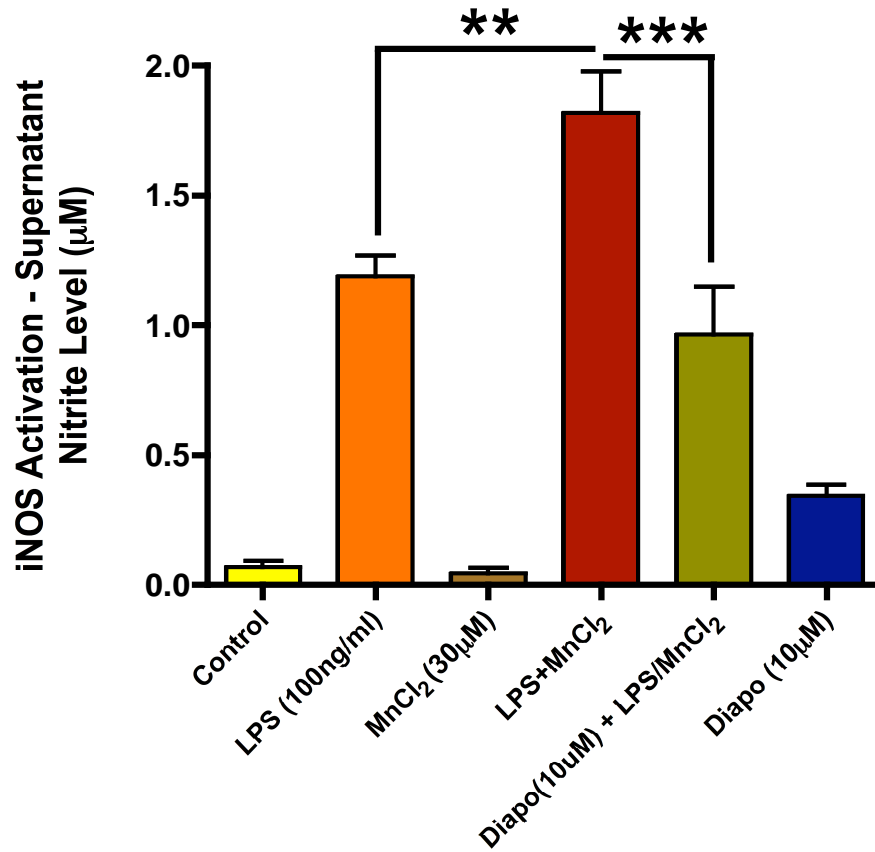


Figure 2. LPS/Mn-induced iNOS activity can be attenuated by diapoecynin in primary microglia.

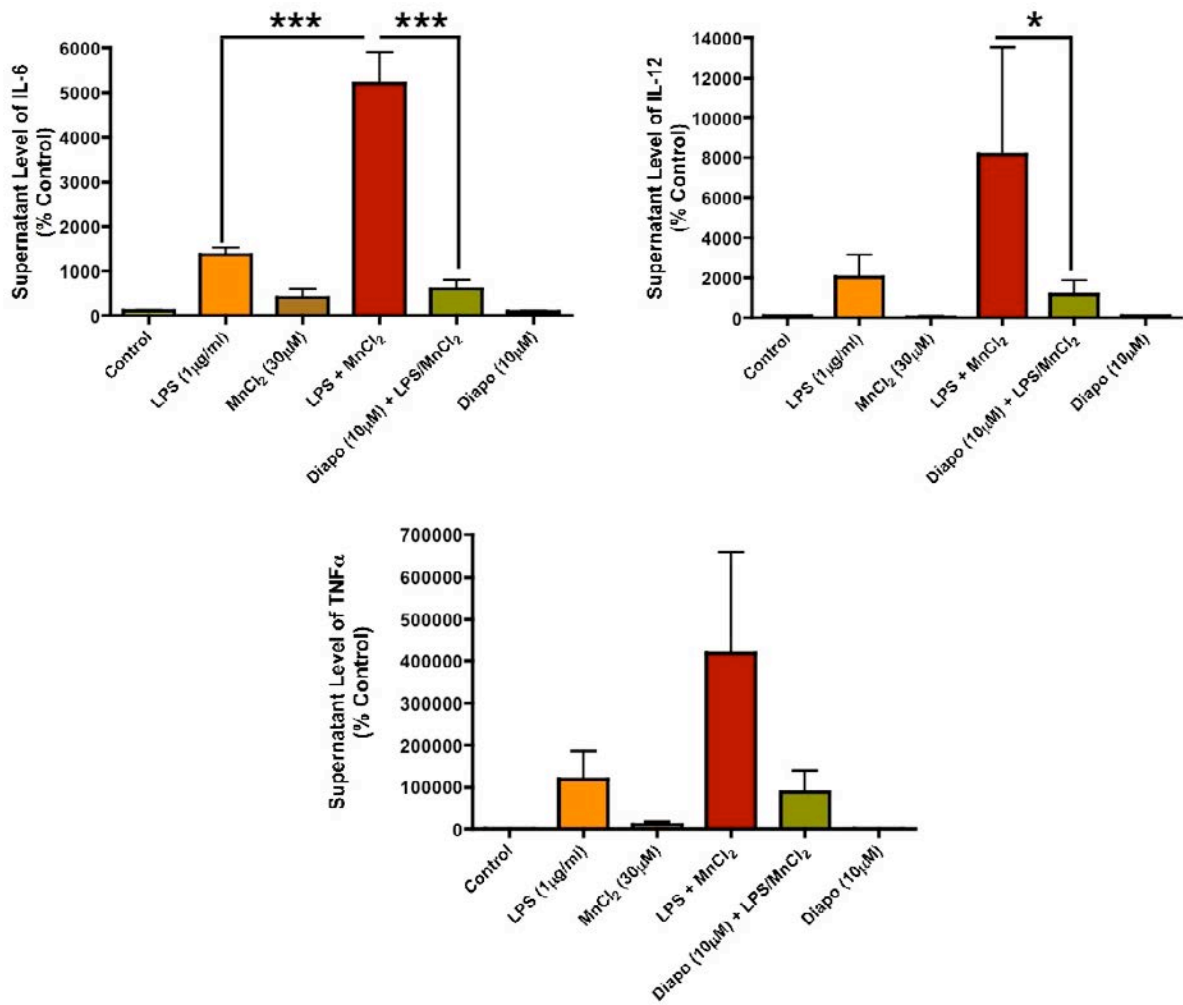


Figure 3. Diapocynin appears to attenuate cytokine release in BV2 cells.

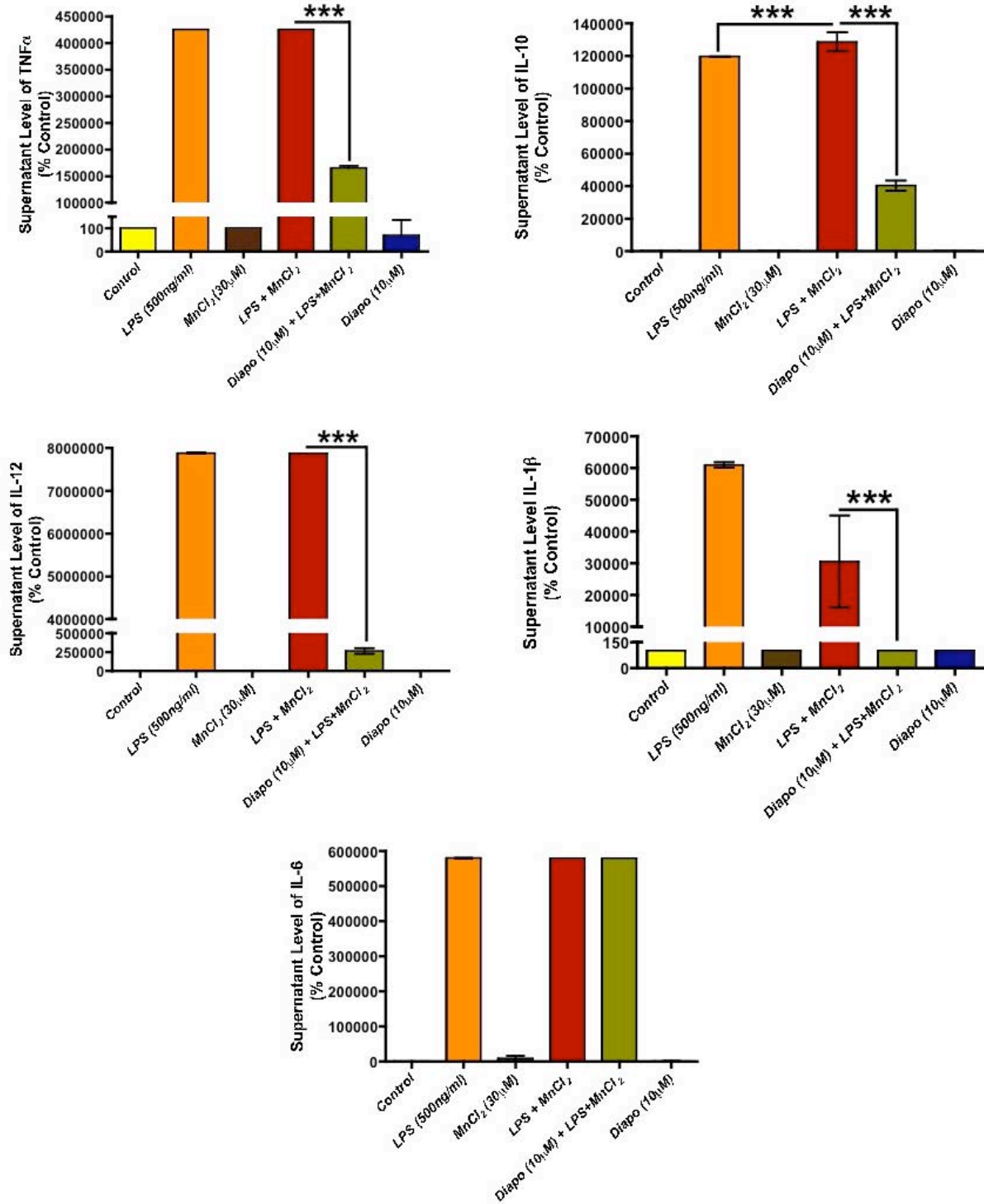


Figure 4. Diapocynin significantly attenuates cytokine release (except IL-6) in primary microglia.

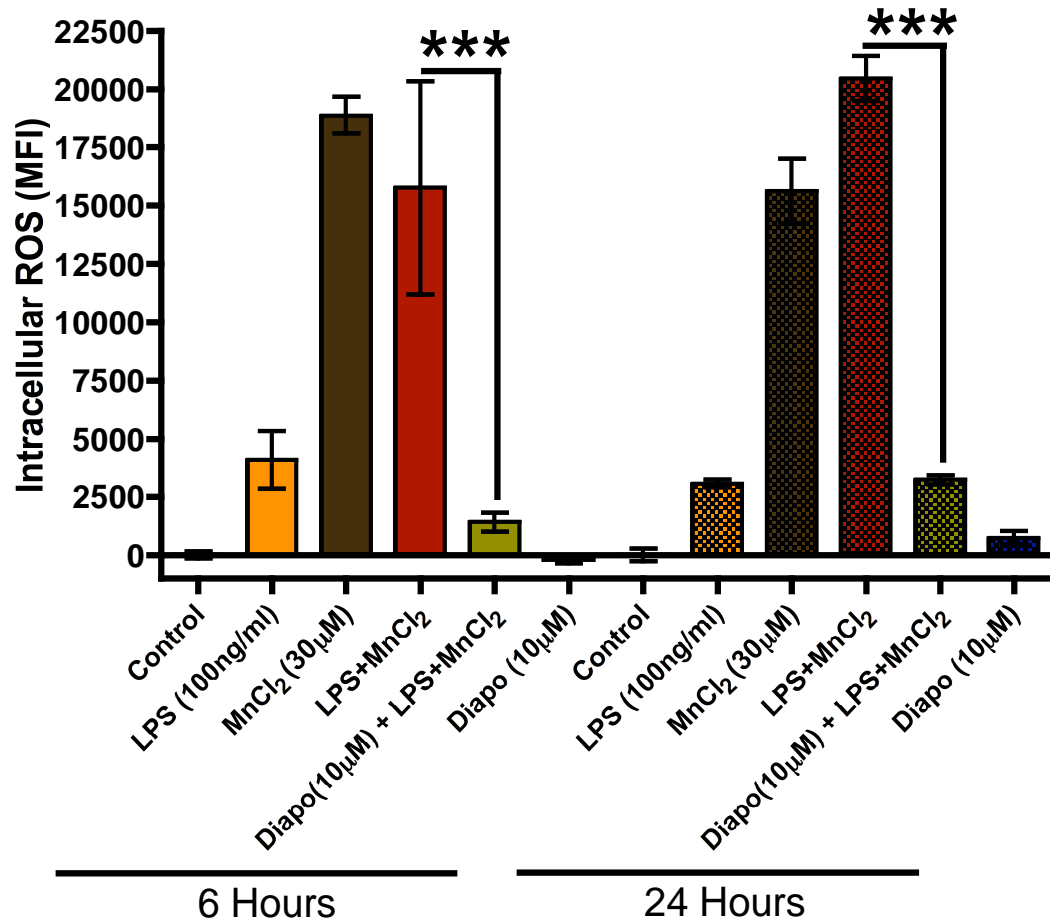


Figure 5. Diapocynin is able to inhibit iROS production at both 6 and 24 hours in primary microglia.

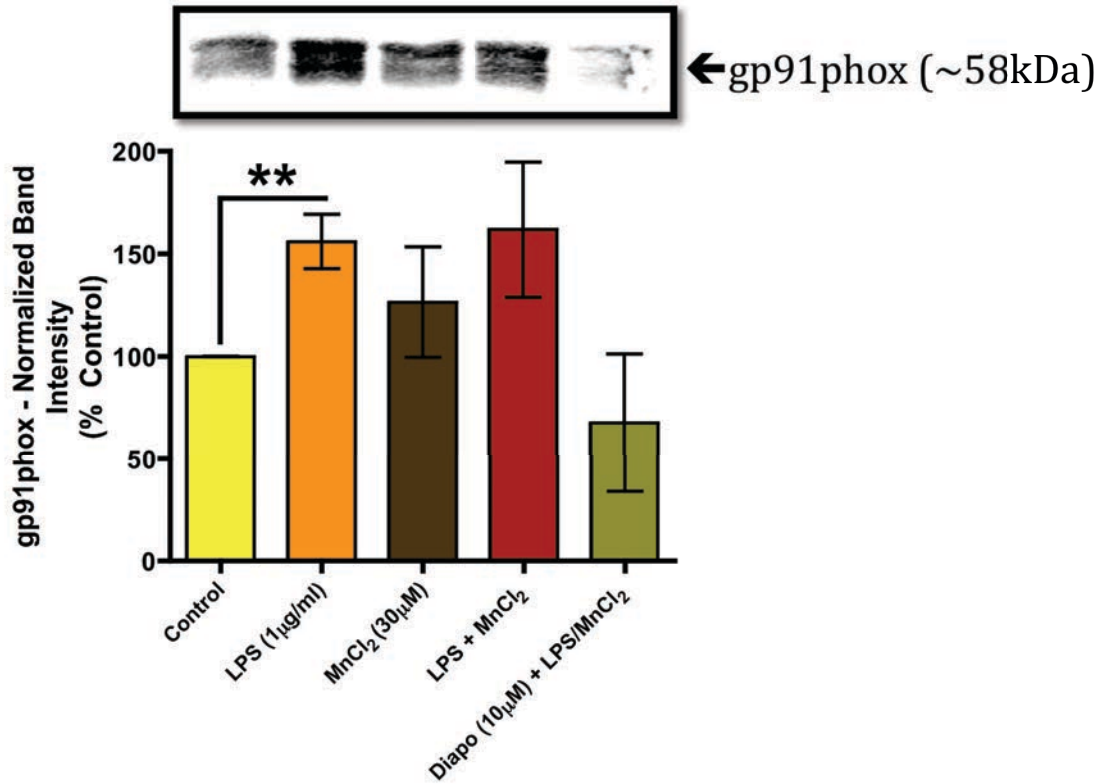


Figure 6. Shows the trend of gp91phox protein expression with diapocynin pretreatment.

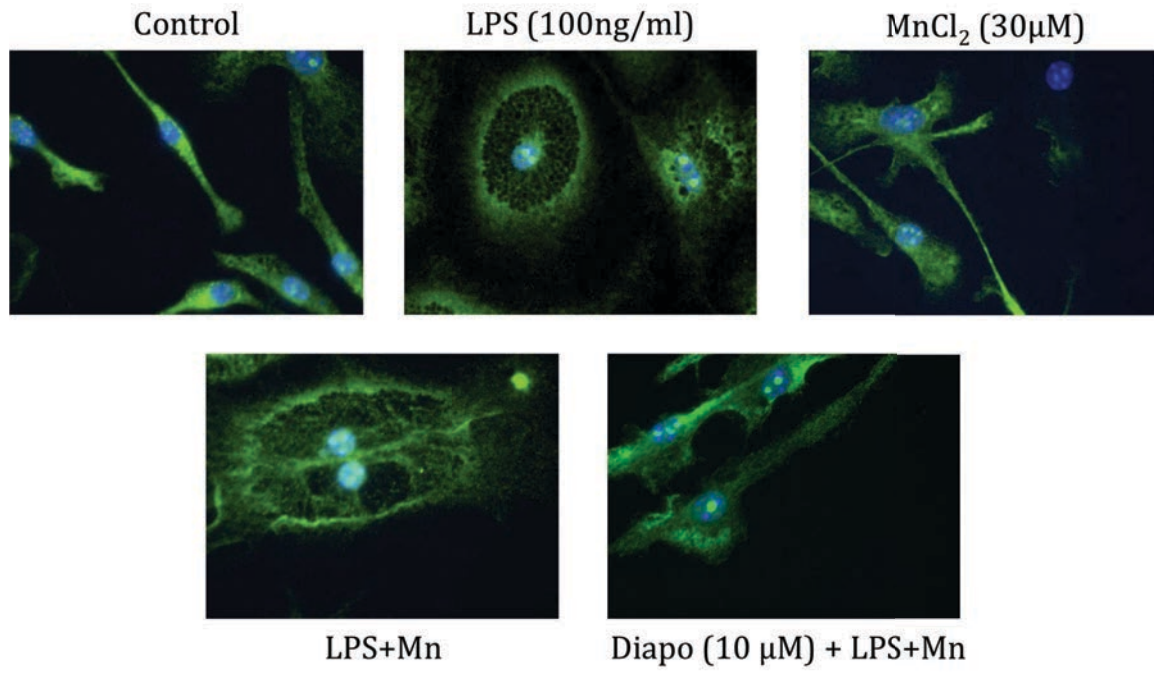


Figure 7. Immunocytochemistry shows microglial morphological changes as well as protein expression of p67phox.

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CHAPTER V: GENERAL CONCLUSION

The etiologies of numerous neurodegenerative diseases have yet to be elucidated, but neuroinflammation has gained recognition as a critical contributing factor to neurodegeneration. Neuroinflammatory markers have become a ubiquitous finding in patients as well as experimental models of PD. These markers are similarly observed in Alzheimer's disease, Huntington's disease, and ALS (Streit, Mrak et al. 2004; Whitton 2007). Therefore, elucidating the complete neuroinflammatory mechanisms is considered paramount for development of therapeutic agents against neuroinflammation. Glial cells, namely microglia and astrocytes, make up the brain's innate immune system, though microglia are considered the main contributors to neuroinflammation (Streit and Kincaid-Colton 1995; Whitton 2007). Recently, manganese (Mn) was shown to modulate and, in some cases, potentiate LPS-induced inflammation (Filipov, Seegal et al. 2005; Chen, Ou et al. 2006). In conjunction with the aforementioned findings, the present studies used a microglia cell culture model of neuroinflammation.

In the initial study, we investigated whether PKC δ plays a role in LPS/Mn-induced inflammation. We first determine that low doses of manganese, alone, are not sufficient to elicit an inflammatory response in microglia cell cultures. However, manganese in combination with LPS induces a robust response, including increases in nitrite and iROS production, and the release of various cytokines. Some of these responses show Mn potentiation of LPS, as seen with nitrite production. After

establishing that manganese and LPS cause inflammation without killing the cells, we wanted to determine if PKC δ played any role in the elicited inflammatory response.

For the initial experiments, we utilized rottlerin, a pharmacological inhibitor of PKC δ . When cells were pretreated with rottlerin, LPS and manganese were unable to mount an inflammatory response, similar to the effect in cells that had not been subjected to rottlerin pretreatment. To determine if the nitrite production observed was a result of iNOS activity, NOS2 protein expression was quantified. We found LPS treatment alone was unable to increase protein expression significantly, though there was an increase in the mean expression, but Mn potentiation caused a significant increase in NOS2 protein expression. Rottlerin pretreatment significantly attenuated the protein expression increase seen following LPS/Mn treatment, which follows the observed trend of nitrite production. To further solidify PKC δ 's role in LPS/Mn-induced nitrite production, we performed *ex vivo* experiments on microglia cultured from wild-type and PKC δ knockout mice. Following the trends seen with rottlerin inhibition of PKC δ , we saw a significant decrease in nitrite production in the absence of PKC δ .

Oxidative stress is particularly toxic to dopaminergic neurons making it an important therapeutic target (Jenner and Olanow 1998). We observed intracellular ROS (iROS) production to determine a role for PKC δ . Mn treatment elicits a robust production of hydrogen peroxide, more so than LPS treatment alone. Treatment with both LPS and Mn caused an even greater production of iROS, which was blocked when cells were pretreated with rottlerin. To further solidify these findings, we used an *ex vivo* model, culturing microglia from wild-type and PKC δ knockout mice. We found a

near complete block of iROS production in the absence of PKC δ . In addition, no difference was observed between treatments, showing Mn is unable to modulate LPS iROS production stimulation when PKC δ is not present.

Cytokines play an important role in the immune response. Using rottlerin as a PKC δ inhibitor, we were able to block the release of TNF α , IL-6, IL-10, and IL-12, but saw no change in the supernatant levels of IL-1 β . We repeated this experiment using microglia cultured from wild-type and PKC δ knockout animals. With the PKC δ gene completely removed, we saw similar results, except IL-1 β release was also attenuated.

These results demonstrate that PKC δ plays an important role in LPS/Mn-induced neuroinflammation. Blocking PKC δ activity greatly decreases proinflammatory events in microglia, while completely knocking out the PKC δ gene further decreases the same proinflammatory events. Collectively, these results indicate PKC δ is a potential therapeutic target against neuroinflammatory events.

Next, we wanted to determine the therapeutic efficacy of a novel NADPH oxidase compound, diapocynin. Apocynin, a naturally occurring methoxy-substituted catechol, has been used experimentally as an NADPH oxidase inhibitor (Simons, Hart et al. 1990; Stefanska and Pawliczak 2008). The NADPH oxidase complex is the main contributor to ROS production, and therefore an attractive target molecule in the prevention of inflammation. Apocynin converts into a dimer through peroxidase-mediated oxidation, to become an active metabolite (Stefanska and Pawliczak 2008). Our laboratory synthesized diapocynin through oxidative coupling of apocynin. We hypothesized that

the already dimerized form of apocynin would be a more potent inhibitor of proinflammatory events caused by LPS insult.

We found diapocynin more efficiently blocked LPS-induced iNOS activation, with an EC_{50} of 6.80 μ M compared to apocynin's EC_{50} of 54.37 μ M. We showed diapocynin is a more effective inhibitor of nitrite production and cytokine release than apocynin. Diapocynin attenuated LPS-induced increases in gp91phox protein expression, while apocynin had no effect on protein expression. Diapocynin also reduced LPS-induced iROS production to below basal levels.

Though the mechanism through which diapocynin acts has yet to be elucidated, we show that diapocynin is a more efficacious inhibitor of LPS-induced proinflammatory events as compared to apocynin. Diapocynin effectively inhibits LPS-induced nitrite production, iROS production, and the release of IL-1 β , IL-10, IL-12, and TNF α . Collectively these data suggest diapocynin is a more potent NADPH oxidase inhibitor than apocynin, and has the potential to be a therapeutic agent against neuroinflammation.

Next we determined the ability of diapocynin to attenuate LPS/Mn-induced neuroinflammatory events. Since Mn has the ability to modulate proinflammatory events caused by LPS insult, we wanted to establish if diapocynin is still an effective inhibitor of nitrite release, iROS production, and cytokine release when cells are cotreated with LPS and Mn. We hypothesized that diapocynin can dampen microglia induced inflammatory events.

Initially, we wanted to assess diapocynin's ability to attenuate LPS/Mn-induced iNOS activation. We observe diapocynin effectively blocks LPS/Mn-induced iNOS activation, which follows the trend seen in the previous studies investigating LPS alone. We observed these results in both BV2 cells and primary microglia. We then assayed for cytokines using both the BV cell and primary microglia cell models. Diapocynin attenuated LPS/Mn-induced release of IL-6 and IL-12 significantly; however, TNF α release was not attenuated significantly, though we did see a large decrease in the mean cytokine release. BV2 cells show great variation between stocks, leading to variation between replicates and increased error bars, which could be the reason no significance was observed between treatment groups. Diapocynin did, however, significantly block the release of IL-1 β , IL-10, IL-12, and TNF α in the primary microglia cell model. No change was seen in IL-6 release with diapocynin pretreatment.

Intracellular ROS production was measured at both 6 hours and 24 hours. Diapocynin pretreatment attenuated LPS/Mn-induced iROS production at each time-point, showing that its anti-inflammatory effects begin early and are sustained throughout the treatment. Along with iROS production, we assessed gp91phox protein expression. Though we did not find any significant changes in gp91phox protein expression across the different treatment groups, we observed a trend in the mean protein expressions. The trend shows an increase in gp91phox protein expression with LPS/Mn treatment, which is attenuated with diapocynin pretreatment.

Using primary microglia, we also visualize changes in cell morphology caused by LPS/Mn insult. In the control group we observe a morphology that is more consistent

with microglia in their 'resting state'. The LPS and LPS/Mn treated microglia show an amoeboid-like morphology consistent with morphology seen in activated microglia, which is expected. Pretreating the cells with diapocynin appears to attenuate these morphological changes, leaving cells that more closely resemble the morphology of the control group cells. This lends support to the hypothesis that diapocynin can block microglial activation.

This study demonstrates that the novel compound diapocynin has the ability to attenuate microglial induced inflammatory responses elicited by treatment with LPS and Mn. We show diapocynin's ability to block the main neuroinflammatory characterizing factors: cytokine release and ROS and RNS production. Collectively, these results indicate that diapocynin may have potential as a therapy against neuroinflammation.

Herein, we have discovered a potential therapeutic target for neuroinflammatory events in PKC δ . We implicate PKC δ in various LPS/Mn-induced microglia inflammatory responses by demonstrating that the absence of PKC δ leads to less robust inflammatory events. In addition, we have characterized the anti-inflammatory effects of a novel compound, diapocynin. We show diapocynin is a more effective anti-inflammatory compound than its parent compound, apocynin. We also show diapocynin can effectively block LPS/Mn-induced microglia inflammatory responses.

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