THE MECHANISM FOR PARAQUAT TOXICITY INVOLVES OXIDATIVE STRESS AND INFLAMMATION: A MODEL FOR PARKINSON'S DISEASE

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THE MECHANISM FOR PARAQUAT TOXICITY INVOLVES OXIDATIVE STRESS AND INFLAMMATION: A MODEL FOR PARKINSON'S DISEASE

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LIST OF ABBREVIATIONS

BH_4	Tetrahydrobiopterin
CNS	Central nervous system
DCF-DA	Dichlorodihydrofluorescein diacetate
DDC	Sodium diethyldithiocarbamate trihydrate
DHE	Dihydroethidium
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DPI	Diphenylene iodonium
ERK	Extra-cellular signal regulated kinase
DAG	Diacylglycerol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GABA	Gamma-aminobutyric acid
IL	Interleukin
IFN	Interferon
iNOS	Inducible nitric oxide synthase
JAK	Janus kinases
JNK	c-Jun-N-terminal kinase
KRH	Krebs-Ringer-Hepes
LDH	Lactate dehydrogenase
LPS	Lipopolysacharide
MAPK	Mitogen-activated protein kinase

MEK	Mitogen-activated protein kinase kinase
MEKK	Mitogen-activated protein kinase kinase kinase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Message ribonucleic acid
MTT	2',7'- 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NO	Nitric oxide
NOS	Nitric oxide synthase
NADPH	Nicotine amide adenine diunucleotide phosphate
NF-κB	Nuclear factor-kappaB
6-OHDA	6-Hydroxydopamine
PD	Parkinson's disease
PKC	Protein kinase C
PQ	Paraquat
RNA	Ribonucleic acid
ROS	reactive oxygen species
SNAP	S-Nitroso-N-acetyl-DL-penicillamine
STAT	Signal transducers and activators of transcription
TNF	Tumor necrosis factor
UV	Ultraviolet

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder known to affect the dopaminergic neurons in the substantia nigra. Epidemiological studies have shown an increased risk of developing PD with exposure to paraquat. Paraquat inhibits complex I of the mitochondrial respiration chain and generates of oxidative stress. In this study, we examined the source of reactive oxygen species (ROS) and the underlying signaling pathway for paraquat-induced cytotoxicity to BV-2 microglial cells. Paraquat-induced ROS production (including superoxide anion) in BV-2 cells was accompanied by translocation of $p67_{phox}$, the cytosolic subunit of NADPH oxidase, to the membrane. Paraquat-induced ROS production was inhibited by NADPH oxidase inhibitors, apocynin and diphenylene iodonium (DPI), but not the xanthine/xanthine oxidase inhibitor, allopurinol. Apocynin and DPI also rescued cells from paraquat-induced toxicity. Inhibitors for either protein kinase C (PKC) or extracellular signal-regulated kinases (ERK1/2) could partially attenuate paraquat-induced ROS production and cell death; a combination of these two kinase inhibitors also partially attenuated paraquat-induced cytotoxicity. Rottlerin, a selective PKCδ inhibitor, also inhibited paraquat-induced translocation of $p67_{phox}$.

Under inflammatory conditions, microglial cells respond to interferon-γ (IFNγ) and lipopolysaccharides (LPS) by induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO). Because paraquat can cause cytotoxicity by increasing ROS production in microglial cells, we also tested whether paraquat may exacerbate cytotoxicity in the presence of NO produced by pro-inflammatory cytokines. Our results show that paraquat greatly inhibited cytokine-induced production of NO induced by both IFNγ and LPS but only moderately enhanced ROS production and cell cytotoxicity in microglial cells. Paraquat-induced attenuation of NO production was not due to either (1) inhibition of iNOS expression, (2) a direct interaction between paraquat with nitrite in the culture medium, or (3) interaction with NO produced by SNAP, an NO donor. Inhibition of NO production by L-Nil, a specific inhibitor for iNOS, did not decrease ROS production by paraquat. On the other hand, examination of nitrotyrosine, an indicator of protein adduct due to peroxynitrite formation, indicated increase due to IFNγ and IFNγ with paraquat. Addition of NADPH, cofactor for both NADPH oxidase and iNOS, did not increase paraquat-induced ROS production but inhibited IFNγ-induced NO production.

We wanted to determine the role of NADPH oxidase in neuronal cells. SH-SY5Y human neuroblastoma cells become dopaminergic neuronal-like after differentiation with retinoic acid. SH-SY5Y cells express the NADPH oxidase subunit $p67_{phox}$ at lower amounts than microglial cells. Paraquat did induce the translocation of $p67_{phox}$ to the membrane. Paraquat also induced the upregulation of the $p67_{phox}$ subunit protein and NOX2 mRNA. It has been shown that increased gene expression of the cytosolic subunits can result in a dramatic increase in the generation of superoxide. Moreover, the low

levels of paraquat that people are generally exposed to can upregulate gene expression of NADPH oxidase and increase superoxide production in neurons.

In conclusion, our data suggest that NADPH oxidase plays a significant role in the toxicity of paraquat, especially in microglia cells that may destroy the substantia nigra and lead to the development of PD. A better understanding of the underlying cause of this disease would help us to develop better strategies and pharmaceutical agents for prevention and treatment of PD.

CHAPTER 1

Oxidative and inflammatory pathways in Parkinson's disease

This chapter will be submitted as a review.

Oxidative and inflammatory pathways in Parkinson's disease

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Abstract:

Parkinson's disease (PD) is the second most prevalent age-related neurodenerative diseases. Patients' pathologically manifests tremors, bradykinesia, abnormal postural reflexes, rigidity and akinesia. Histology revealed landmark losses of the dopaminergic neurons in the substantia nigra. Although the etiology of PD has been intensively pursued for several decades, clear answers to explain the specific destruction of these nigral neurons have started to be elucidated the last few years. Both genetic and epigenetic factors are implicated in the initiation and progression of the disease. For the latter, specific drug contaminates (MPTP) and environmental toxins (paraquat and rotenone) have been shown to increase the risk of PD in humans. However, these toxins were found to have far less potent actions on neurons than neighboring microglia cells (macrophage-like cells in the brain). Moreover, microglia are recruited to this area of the brain during chemical or infectious insult. Recent studies with cell and animal models revealed that these toxins to activate signaling pathways that produce reactive oxygen species (ROS) and inflammatory responses in glial cells to subsequently destroy neighboring dopaminergic neurons. Because oxidative stress remains the leading theory explaining progression of PD, this review describes pathological effects of neurotoxins on cells and subcellular mechanisms for producing ROS that almost certainly will provide ways to prevent and treat PD in the future.

Oxidative Stress and neurodegenerative diseases:

Many cellular reactions utilize molecular oxygen for catalysis and energy production. These reactions in turn produce reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals; peroxy radicals and reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite are also produced. While these reactive species are important for execution of physiological functions, excessive production is detrimental to cell membranes and can cause cell death (Loh et al., 2006). To cope with the many oxidative reactions, antioxidants such as glutathione and vitamin E as well as antioxidant enzymes such as superoxide dismutases, catalases and glutathione peroxidase are present in young people to balance the oxidative mechanisms. Oxidative stress in cells due either to excessive production of ROS or insufficient antioxidant defense particularly in the elderly can damage cellular molecules including proteins, lipids and DNA and activate apoptotic pathways (Betarbet et al., 2005; Loh et al., 2006; Mariani et al., 2005; Mattson, 2006).

The brain is only 2-3% of the total body mass, but it consumes 20% of basal oxygen (Mariani et al., 2005; Moreira et al., 2005). Cells in the brain are particularly susceptible to oxidative damage due to the high concentrations of polyunsaturated fatty acids in their membranes and relatively low activity of endogenous antioxidant enzymes (Mariani et al., 2005). Aging is associated with an increased oxidative stress and accumulation of oxidative damage to biomolecules which gradually weakens cognition (Cardoso et al., 2005; Mariani et al., 2005). More oxidized proteins, including carbonyls and nitro-protein adducts are found in the brains of elderly (Mariani et al., 2005). High

levels of 8-hydroxy-2-deoxy-guanosine, a marker of oxidized DNA, were detected in both nuclear and mitochondria DNA in elderly brains (Mariani et al., 2005). The increase in oxidative stress together with the decline in endogenous antioxidants are important underlying risk factors in the brains of elderly for development of neurodegenerative diseases including Alzheimer's and Parkinson's disease (Cardoso et al., 2005; Mariani et al., 2005).

Parkinson's disease:

Over one million people in the United States are affected by PD (Chun et al., 2001). PD, a movement disorder, is the second most common neurodegenerative disease in the elderly; it generally affects people over 55 years of age (Schober, 2004; Siderowf and Stern, 2003). This disease is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta and a gradual accumulation of Lewy bodies which are deposits of specific cytoplasmic proteins such as ubiquitin, α-synuclein, and oxidized neurofilaments (Gao et al., 2003a; Gelinas and Martinoli, 2002; Pesah et al., 2004; Peng et al., 2005; Pesah Y., 2004; Thiruchelvam et al., 2000). Dopaminergic neurons are necessary for normal motor function; loss of the neurons causes resting tremors, bradykinesia, abnormal postural reflexes, rigidity and akinesia (Pesah et al., 2004; Uversky, 2004). By the time symptoms appear, 50- 80% of the dopaminergic neurons have been lost (Schober, 2004; Uversky, 2004). Normally there are around 400,000 pigmented nerve cells in the substantia nigra (Uversky, 2004). With normal aging approximately 2,500 of these nerve cells are lost every year. Therefore a person should not develop Parkinson's disease until at least 130 years old (Uversky, 2004).

Mechanism(s) that account for the accelerated loss of dopaminergic neurons in PD patients remain elusive (Tieu, 2003).

Post mortem studies demonstrated increased oxidation of proteins, lipids, and DNA in neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, stroke, progressive supranuclear palsy, Huntington's disease, Creutzfeldt-Jakob disease, and amyotrophic lateral sclerosis (Ahlskog, 2005; Beal, 2004; Cardoso et al., 2005; Gandhi and Wood, 2005; Hald and Lotharius, 2005; Mariani et al., 2005; Uversky, 2004). Dopaminergic neurons oxidize dopamine by monoamine oxidase, a reaction known to cause production of superoxide and hydrogen peroxide (Betarbet et al., 2005; Cardoso et al., 2005; Greenamyre et al., 2001; Mariani et al., 2005; Maguire-Zeiss et al., 2005). Consequently, dopaminergic neurons are in a perpetual state of oxidative stress, and this imbalance may lead to reduced levels of endogenous antioxidants (Beal, 2004; Cardoso et al., 2005; Gandhi and Wood, 2005;Greenamyre et al., 2001).

Herbicide use and PD:

Epidemiological studies have identified several risk factors for PD including using well water, living on a farm, occupational use of herbicides, family history of PD, and head trauma (Firestone et al., 2005; Semchuk et al., 1993). Among these, the greatest risk for developing PD is age, followed by family history, head injuries and herbicide use. Mutations in α-synuclein and parkin have been shown in a small fraction of early onset familial Parkinson's disease (Chun et al., 2001). It is unclear whether the familial component of PD reflects genetic reasons or exposure to the same environmental toxins, but most likely it is the combination of both (Semchuk et al., 1993). The majority

of patients do not report a positive family history of PD, suggesting that nongenetic factors are more important risk factors (Semchuk et al., 1993). Non-familial PD occurs after the age of 50 and is over 90% of cases (Chun et al., 2001; Gelinas and Martinoli, 2002; Shimizu et al., 2003b; Siderowf and Stern, 2003). Twin studies have shown head trauma to be a significant risk factor (Semchuk et al., 1993). A twin that had suffered a head injury was twice as likely to develop PD as their healthy twin (Semchuk et al., 1993). An increased risk for Parkinson's disease is correlated with exposure to environmental factors including herbicides containing paraquat, but there was no increased risk with occupational exposures to chemicals in general, heavy metals, and minerals (Brooks et al., 1999; Liou et al., 1997; Yang and Sun, 1998b) . However, despite a three-fold increase in PD diagnosis, only 10% of patients reported using any herbicide (Semchuk et al., 1993).

Neurotoxins producing PD-like symptoms:

6-Hydroxydopamine:

6-Hydroxydopamine (6-OHDA) was the first dopaminergic neurotoxin discovered and has been used experimentally in models of PD for over 30 years (Bove et al., 2005; Uversky, 2004). This analog of dopamine and norepinephrine can be taken up by catecholaminergic neurons through the dopamine and norepinephrine transporters (Bove et al., 2005; Schober, 2004). 6-OHDA can damage catecholaminergic neurons throughout the body; it even depletes norepinephrine in sympathetic nerves in the heart (Bove et al., 2005; Schober, 2004). This compound not only is toxic to neurons but also can induce activation of glial cells (Bove et al., 2005). Like dopamine, 6-OHDA does not

readily cross the blood-brain barrier, but it does accumulate in the brain if injected directly into the ventricles (Bove et al., 2005; Schober, 2004). Interestingly, accumulation of endogenous 6-OHDA has been shown in PD patients (Schober, 2004). Upon transport to the neurons, 6-OHDA is oxidized like dopamine to generate free radicals and quinones (Bove et al., 2005; Schober, 2004). 6-OHDA inhibits mitochondrial complex I and produce superoxide and hydroxyl radicals through participation in the Fenton reaction (Schober, 2004). Although administration of 6-OHDA produces many symptoms resembling those in PD, it is worth noting that treatment with this compound does not cause the formation of Lewy bodies (Bove et al., 2005; Uversky, 2004).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP):

In 1982, several drug users in Northern California developed severe Parkinson-like symptoms from a contaminant in synthetic heroin, MPTP (Bove et al., 2005; Landrigan et al., 2005; Schober, 2004; Wersinger and Sidhu, 2006). This discovery gave the first indication that exogenous toxins may be involved in the pathophysiology of PD (Wersinger and Sidhu, 2006). MPTP-intoxicated individuals exhibit clinical neurological features of PD including tremor, rigidity, slowness of movement, postural instability, and freezing (Bove et al., 2005; Brown et al., 2006; Schober, 2004). As in PD, patients who died also show, upon autopsy, damage to the dopaminergic system in the substantia nigra, but unlike PD, Lewy bodies were not found (Bove et al., 2005; Landrigan et al., 2005; Schober, 2004).

MPTP is a lipophilic molecule that readily crosses the blood-brain barrier. This parent compound is not toxic to dopaminergic neurons, but is readily metabolized into a more toxic metabolite (Schober, 2004; Uversky, 2004). In astrocytes, monoamine oxidase type B first converts MPTP into MPDP and then oxidizes it to a pyridinium ion (MPP^+), a toxic metabolite (Maguire-Zeiss et al., 2005; Schober, 2004; Wersinger and Sidhu, 2006). The resulting polar metabolite is released from astrocytes and is taken up into dopaminergic neurons (Schober, 2004). MPP^+ binds to the dopaminergic transporter with high affinity and mice lacking this transporter fail to exhibit toxicity to MPTP (Maguire-Zeiss et al., 2005; Schober, 2004; Uversky, 2004). Normal dopaminergic neurons accumulates MPP^+ to high levels in the mitochondria where it inhibits their electron transport chain complex I as ROS are generated (Bove et al., 2005; Greenamyre et al., 2001; Kotake and Ohta, 2003; Maguire-Zeiss et al., 2005; Schmidt and Alam, 2006; Schober, 2004; Uversky, 2004). MPP^+ is also transported into synaptic vesicles through the vesicular monoamine transporter that stores dopamine (Greenamyre et al., 2001; Schober, 2004). This uptake system serves as a detoxification mechanism since mice with a significant depletion of the vesicular monoamine transporter are more sensitive to MPP^+ toxicity because all of MPP^+ then goes to the mitochondria (Schober, 2004). In dopaminergic cell lines, MPP^+ produces ROS in mitochondria which then triggers the classic apoptosis cascade including caspase activation and DNA fragmentation (Kotake and Ohta, 2003). MPTP toxicity shares many similarities to PD except Lewy bodies are absent (Bove et al., 2005; Landrigan et al., 2005; Schober, 2004). The accidental intoxication of drug abusers with MPTP in the early 1980's demonstrated that even

limited exposure of this compound can result in neurodegeneration that is both self perpetuating and long lasting (Landrigan et al., 2005).

Paraquat:

Paraquat (PQ), 1,1'-dimethyl-4,4-bipyridinium, is a widely used herbicide in the 20th and early 21st centuries (Brooks et al., 1999; Wesseling et al., 2001; Yang and Sun, 1998b). PQ is a cationic non-selective herbicide used to control weeds and grasses in agriculture fields such as cotton (Shimizu et al., 2003a; Thiruchelvam et al., 2000; Yang and Tiffany-Castiglioni, 2005). Unfortunately, the waste greenery from cotton is fed to beef cattle (Thiruchelvam et al., 2000). PQ, regulated in the United States, is considered safe by industry and worldwide regulators, but some of the long-term effects of paraquat include Parkinson's disease, lung damage, and skin cancer (Brooks et al., 1999; Wesseling et al., 2001; Yang and Sun, 1998b). There is significant overlap between geographic areas where paraquat is used and the prevalence of PD (Shimizu et al., 2003b). People exposed to paraquat develop Parkinson-like symptoms. Paraquat is structurally similar to MPP^+, the active metabolite for MPTP (Brooks et al., 1999; Chun et al., 2001; Peng et al., 2005; Shimizu et al., 2003b; Thiruchelvam et al., 2000; Yang and Sun, 1998b). Epidemiological data have shown that exposure to MPP^+ or paraquat are risk factors for Parkinson's disease (Chun et al., 2001). Paraquat and MPTP deplete dopaminergic neurons in substantia nigra and cause the symptoms of Parkinsonism (Brooks et al., 1999; McCormack et al., 2002; Thiruchelvam et al., 2000; Wesseling et al., 2001; Yang and Sun, 1998a). In fact, a study by Kang et al. (1997) showed that paraquat is more cytotoxic than MPP^+.

Despite being a polar molecule, paraquat has been shown to accumulate in the brain (Chun et al., 2001) . It crosses the blood-brain barrier, mainly through the neutral amino acid transporter (Brooks et al., 1999; Chun et al., 2001; Thiruchelvam et al., 2000; Yang and Sun, 1998b). L-valine can competitively inhibit the transport of paraquat across the blood brain barrier by the neutral amino acid transporter (McCormack and Di Monte, 2003; Shimizu K., 2003). One in the brain, paraquat is selectively taken up at the nerve terminals of dopaminergic melanin-containing neurons in substantia nigra through the dopamine transporter (Brooks et al., 1999; Chun et al., 2001; Shimizu et al., 2003a; Thiruchelvam et al., 2000; Yang and Sun, 1998b; Yang and Tiffany-Castiglioni, 2005). Therefore, inhibition of dopamine transporter by cocaine and other compounds is protective against paraquat toxicity to dopaminergic neurons (Shimizu et al., 2003a;Yang and Tiffany-Castiglioni, 2005). Paraquat injected intraperitoneally ends up concentrated in the substantia nigra and associated with loss of dopaminergic neurons (Brooks et al., 1999; McCormack et al., 2002). Interestingly, this toxicity is selective since GABAergic cells in the same region were not affected (Brooks et al., 1999; McCormack et al., 2002).

Within neurons, paraquat can produce intracellular ROS leading to production of malondialdehyde, protein carbonyls, and DNA fragmentation (Peng et al., 2005; Thiruchelvam et al., 2005; Yang and Tiffany-Castiglioni, 2005). Paraquat is reduced by NADPH in redox cycling to give rise to paraquat radicals and eventually to hydroxyl free radicals and superoxide anions (Brooks et al., 1999; Kang et al., 1997; Thiruchelvam et al., 2000; Yang and Sun, 1998a). Paraquat inhibits mitochondrial complex I and perturbs the mitochondrial respiration chain causing impaired energy metabolism, proteasomal

dysfunction and intracellular ROS production (Bretaud et al., 2004; Shimizu et al., 2003a; Yang and Tiffany-Castiglioni, 2005).

Rotenone:

One of the most recent approaches to develop cell models to study PD results from the observation that the pesticide rotenone was previously used by Indians to poison fish (Bove et al., 2005; Uversky, 2004). Rotenone is the most potent member of a family of cytotoxic compounds called rotenoids (Bove et al., 2005). Around the world, rotenone is used commonly as an insecticide and pesticide (Bove et al., 2005; Kotake and Ohta, 2003; Uversky, 2004). Rotenone is used in vegetable gardens to kill insects and in lakes to kill fish that are invasive and overpopulated (Kotake and Ohta, 2003; Uversky, 2004). To date, treatment with rotenone in the USA is restricted to times outside when crops are grown and people are swimming (Bove et al., 2005). Rotenone is a naturally occurring complex ketone extracted from the roots of Lonchocarpus species (Kotake and Ohta, 2003; Uversky, 2004). Rotenone is easily degraded by exposure to sunlight, soil and water. Therefore, the likelihood of environmental exposure to rotenone is very low, which make it advantageous as a pesticide (Bove et al., 2005; Uversky, 2004).

Ingestion of rotenone is not likely to have adverse effects in healthy people. It is slowly and incompletely absorbed in the stomach and intestines and then efficiently degraded by the liver's first pass effect (Bove et al., 2005). Thus, one would have to either ingest a large amount of this compound or have a liver disease in order for rotenone to exceed the liver's ability to detoxify only then would it enter the general circulation (Bove et al., 2005). Once rotenone enters the body, like MPTP, it is very

lipophilic and crosses the blood-brain barrier to distribute evenly throughout in the brain (Bove et al., 2005; Uversky, 2004).

Since 1950s, it is believed that rotenone acts as an inhibitor for mitochondria (Kotake and Ohta, 2003). Because it is very lipophilic, rotenone not only distributes through the body and brain, it crosses into all organelles including double-membrane encased mitochondria where it inhibits with high affinity complex I of the mitochondrial electron transport chain (Bove et al., 2005; Kotake and Ohta, 2003; Maguire-Zeiss et al., 2005; Uversky, 2004). Fortunately, most cells are able to detoxify the oxidative damage caused by rotenone using their antioxidant protective enzyme pathways. Unfortunately, chronic inhibition of this mitochondrial complex can cause selective degeneration of dopaminergic neurons despite being uniformly distributed throughout the brain (Uversky, 2004). This raises the question why dopaminergic cells are targeted for destruction and whether rotenone in the environment contributes to the etiology of PD in certain individuals. Due to its inhibition of complex I, rotenone is known to cause ROS generation, ATP depletion, and cell death in neurons (Uversky, 2004). In agreement with the involvement of ROS in rotenone toxicity, antioxidants are capable of attenuating rotenone-mediated cell death (Uversky, 2004). Interestingly, rotenone also inhibits the formation of microtubules (Bove et al., 2005). Similar to PD, treatment with rotenone also leads to the aggregation of a-synuclein and formation of Lewy bodies (Bove et al., 2005). Another similarity to PD is that rotenone depletes glutathione a primary cell antioxidant (Uversky, 2004). Thus, rotenone toxicity mimics many pathological hallmarks of PD, including neurodegeneration of dopaminergic neurons in substantia nigra and formation of Lewy bodies (Uversky, 2004). Presumably due to oxidative

damage, and depletion of glutathione and disruption of axonal transport (Bove et al., 2005; Uversky, 2004).

Microglia involvement in oxidative stress:

All known dopaminergic neurotoxins exhibit a common characteristic. They inhibit either mitochondrial complex I or complex III in neurons but at a fairly high concentrations (Schober, 2004). Apparently something must be super sensitizing these cells. Oxidative stress in the CNS comes not only from mitochondrial-generated ROS in neurons but from activated microglia (Chun et al., 2001). Very recently reactive microglia were shown to play a critical role in several neurodegenerative disorders including Parkinson's disease especially if they are recruited to sites within the brain by stimuli such as interferon (Block et al., 2006; Li et al., 2005a; Li et al., 2005b; Qin, 2005; Zhang et al., 2005). Indeed, Parkinson's patients can have more than six times the number of reactive microglia as compared to controls (Arai et al., 2004; McGeer et al., 1988a; McGeer et al., 1988b) . However, it is unknown whether these microglia initiate or aggravate neurodegeneration (Zhang et al., 2005). Clearly stimulation of either microglial or astrocyte activities correlate with neuronal injury induced by paraquat (Chun et al., 2001; McCormack et al., 2002) . Interestingly, a study by Gao et al. (2003b) showed microglia clearly contribute to dopaminergic neurodegeneration caused by MPTP while astrocytes do not. This observation radically altered PD research.

Microglia behave as macrophages in the brain and are one of four major cell types in the CNS (Dringen, 2005; Mander et al., 2006). Similar to macrophages, a major function of microglia is to fight infection and remove debris in the brain (Dringen, 2005).

Microglia become activated and are recruited to areas of brain damage by exposure to immune mediators and chemotactic factors such as IL-1beta or TNF-alpha (Mander et al., 2006). Besides producing cytokines, proteases, and prostanoids, activated microglia also rapidly generate superoxide and nitric oxide to kill invading cells (Dringen, 2005). In order to survive in their own environment of oxidative free radicals, it is not surprising that microglia also contain high levels of endogenous antioxidants including superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and NADPH regenerating enzymes (Dringen, 2005). However, if their antioxidant safety system was slowed by agents known to initiate PD then a realistic explanation for the etiology of PD would be within our grasp.

Activated microglia in the substantia nigra are found in several models for PD, including exposure to MPTP, rotenone, substance P, methamphetamine and infection (Block et al., 2006; Delgado, 2003; Gao et al., 2002; Gao et al., 2003b; Scheller et al., 2005; Thomas et al., 2004; Wu et al., 2003). In many instances, activation of microglia and generation of ROS coincide with neurochemical changes such as a decrease in dopamine synthesis (Scheller et al., 2005; Wu et al., 2003). While some neurochemical changes happen quickly and can be detected before evidence of any lesions, others are delayed and are manifested long after neurotoxin exposure (Barcia et al., 2004). These results imply that a brief exposure to an acute brain insult can initiate a chronic process of neurodegeneration (Langston et al., 1999). Microglia may respond quickly to factors posing a threat to the brain and then contribute to progression of PD and enhance neurotoxicity by neurotoxins (Casarejos et al., 2006; Gao et al., 2002). It is noteworthy that minocycline, an antibiotic, known to slow microglia responsiveness was observed to

attenuate the neurotoxicity of 6-OHDA, MPTP and rotenone (Casarejos et al., 2006; Croisier et al., 2005).

Contribution of oxidative stress by activated NADPH oxidase:

NADPH oxidase is an enzyme highly expressed in microglial cells and other immune cells that metabolizes molecular oxygen and generates superoxide as a product (Wu et al., 2003). There is evidence that NADPH oxidase is upregulated in cells found in the substantia nigra in PD patients. As previously noted, activated microglia are six times more prevalent in the substantia nigra of PD patients (Arai et al., 2004; McGeer et al., 1988a; McGeer et al., 1988b).

NADPH oxidase is comprised of three cytosolic components ($p47_{phox}$, $p67_{phox}$, and $p40_{phox}$) and two membranous subunits ($gp91_{phox}$ and $p22_{phox}$) (Sumimoto et al., 2004). Besides translocation of the cytosolic subunits to the membrane subunits, the enzyme complex also requires a small GTPase Rac for full function (Sumimoto et al., 2004). Translocation of the cytosolic subunits is initiated by phosphorylation by protein kinases. In the $p47_{phox}$ subunit, phosphorylation results in conformational change and the SH3 domain then binds with the $p22_{phox}$ membrane subunit (Sumimoto et al., 2004). Recent studies have identified activated NADPH oxidase as a major source of superoxide in a number of neurodegenerative diseases including PD (Li et al., 2005b). Mice defective in NADPH oxidase show less neurotoxic response to LPS (Qin, 2004), MPTP (Wu et al., 2003), and rotenone (Casarejos et al., 2006). NADPH oxidase appears to be ubiquitously expressed in all brain regions and cell types including neurons and glial cells (Infanger et al., 2006). Much higher levels of this enzyme are found in microglia similar

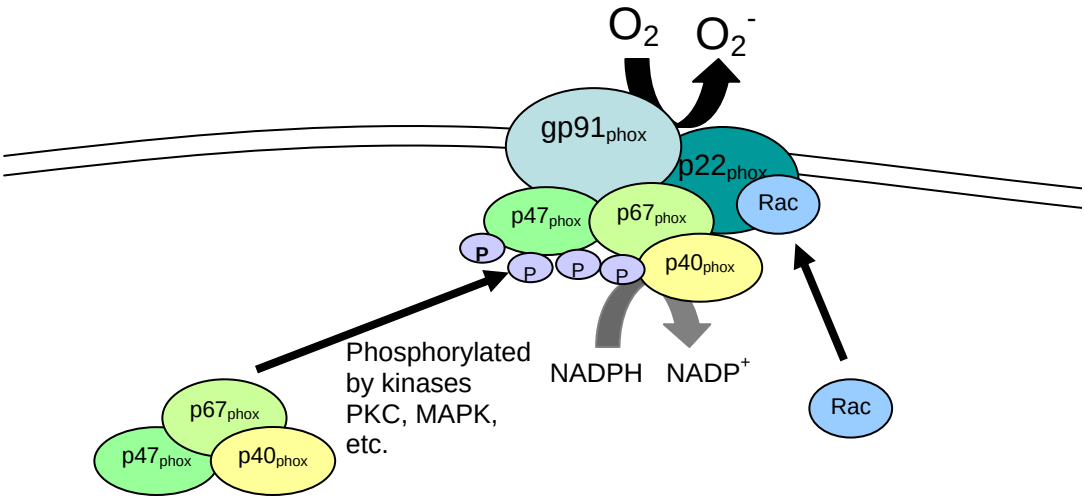

Fig 1. Schematic of activation of NADPH oxidase

NADPH oxidase consists of two membrane bound subunits, $gp91_{phox}$, and $p22_{phox}$, and 3 cytosolic subunits, $p47_{phox}$, $p67_{phox}$ and $p40_{phox}$. There is also a small GTPase associated with the enzyme. The cytosolic subunits must be phosphorylated by kinases such as PKC and MAPK and then translocate to the nucleus in order for the enzyme to be active.

to other immune cells, Recent studies show that paraquat can induce microglia to produce ROS through NADPH oxidase (Bonneh-Barkay et al., 2005) and confer neurotoxicity by damaging neighboring neurons (Wu et al., 2005). Microglia lacking a functional NADPH oxidase in coculture with neurons failed to produce neurotoxicity as compared with microglia having a functional NADPH oxidase (Wu et al., 2005). These observations appear to provide a major advance to direct definitive studies for PD.

Protein Kinase C Activation of NADPH Oxidase:

The more active NADPH oxidase is the more ROS it will generate. Not surprising, this enzyme responds to many factors regulating cellular activity. Several protein kinases including protein kinase C (PKC) and MAPK have been implicated in activation of NADPH oxidase. PKC isoforms are ubiquitously expressed in brain tissue and are involved in regulation of ion channels and receptors, neurotransmitter release, and synaptic development (Ahlemeyer et al., 2002; Battaini, 2001; Catarsi and Drapeau, 1997; Ran et al., 2003; Wagey et al., 2001). Abnormal PKC has been shown in a number of neurodegenerative diseases including PD (Ahlemeyer et al., 2002; Kozikowski et al., 2003; Wagey et al., 2001).

There are more than 10 PKC isoforms in mammalian cells generally grouped as conventional, novel and atypical based on their structure and function. Both the conventional and novel isoforms contain a conserved C1 domain that binds diacylglycerol or phorbol esters (Kozikowski et al., 2003). The conventional PKCs also contain a C2 domain for binding calcium (Kozikowski et al., 2003). The C3 and C4

domains are conserved throughout all the isoforms and contain the ATP binding and the catalyst domains, respectively (Kozikowski et al., 2003). All PKC isoforms contain a pseudosubstrate domain and under normal conditions, the PKC kinase region is autoinhibited by their own pseudosubstrate domain (Battaini, 2001). The conventional and novel PKC are translocated to the membrane while they are activated by diacylglycerol. The presence of several different isoforms of PKC within a cells provides substrate specificity, differential regulation and an array of functions to accommodate various cell activities (Ran et al., 2003).

Protein kinase C delta:

Protein kinase C delta (PKCδ) is a novel isoform of PKC that is independent of calcium unlike conventional isoforms. PKCδ is expressed in many cell types and is involved in the mitochondrial-dependent pathway of apoptosis (DeVries et al., 2002; Gallagher et al., 2001). PKCδ-null transgenic mice have cells that can not mitochondrial-dependent apoptosis (DeVries et al., 2002; Shibukawa et al., 2003). On the other hand, overexpression of PKCδ in certain cell types can exacerbate apoptosis (Cataisson et al., 2003; DeVries et al., 2002) . PKCδ is a substrate for caspase; cleavage by caspase-3 permanently activates PKCδ (Anantharam et al., 2002; Leverrier et al., 2002). Rottlerin, a selective PKCδ inhibitor, can inhibit caspase-3 activation and thereby decrease PKCδ cleavage. In turn, the latter can also activate caspase 3 thus forming a positive feedforward stimulation between caspase-3 and PKCδ (Anantharam et al., 2002; Leverrier et al., 2002). In pheochromocytoma cells, generation of reactive oxygen species (ROS) resulted in proteolytic cleavage of PKCδ, release of cytochrome C from the

mitochondria and activation of caspase 9 and caspase 3 (Anantharam et al., 2002). PKCδ inhibition by prolonged treatment with PMA or pretreatment with rottlerin blocked H_2O_2-induced JNK activation and apoptosis (Shibukawa et al., 2003).

Ample evidence indicates a role for PKCδ in phosphorylation of cytosolic subunits and activation of NADPH oxidase. ROS production is inhibited by rottlerin or dominant negative mutant of PKC (Bey et al., 2004; He et al., 2004; Shao and Nadel, 2005; Talior et al., 2005; Waki et al., 2006). For example, cells deficient in PKCδ failed to generate ROS while overexpression of PKCδ increased the generation of superoxide anion (Talior et al., 2005; Talior I., 2005; Zhao et al., 2005). PKCδ phosphorylates $p67_{phox}$ and $p47_{phox}$ and this phosphorylation of $p67_{phox}$ and $p47_{phox}$ subunits could be inhibited by rottlerin or antisense oligodeoxyribonucleotides specific for PKCδ (Serezani et al., 2005; Zhao et al., 2005). Rottlerin or PKCδ antisense oligodeoxyribonucleotides also inhibited translocation of the subunits to the membrane (Bey et al., 2004) and we showed that rottlerin reduced ROS generation in microglia cells.

Mitogen Activated Protein Kinase activation of NADPH oxidase:

MAPKs are highly conserved and ubiquitously expressed in all tissue and respond to host and environmental stresses (Bardwell, 2006; Nishimoto and Nishida, 2006). They are activated by phosphorylation of neighboring threonine and tyrosine amino acids, and they phosphorylate serine or threonine amino acids that are adjacent to prolines on other proteins (Bardwell, 2006; Cuschieri and Maier, 2005). There are five MAPK families including extracellular-regulated kinase (ERK)1/2; JNK1/2/3; p38α,β,γ,δ; ERK5; and ERK7 (Cuschieri and Maier, 2005; Kaminska, 2005; Nishimoto and Nishida, 2006; Uhlik

et al., 2004). Each MAPK is activated by a MEK (also called MAPK kinase), which is activated by a MEKK (also called MAPK kinase kinase) (Bardwell, 2006; Roux and Blenis, 2004; Uhlik et al., 2004). The GTPase Ras phosphorylates and activates MEKK (Cuschieri and Maier, 2005; Murphy and Blenis, 2006). In total there are at least 11 MAPKs, 7 MEKs and 20 MEKKs (Uhlik et al., 2004). MAPKs activate a wide range of proteins including transcription factors, other kinases and enzymes (Bardwell, 2006; Kaminska, 2005). MAPKs can translocate from the cytosol to the nucleus and affect gene expression (Kaminska, 2005; Murphy and Blenis, 2006; Nishimoto and Nishida, 2006). ERK1/2 are the most researched MAPKs. ERK1/2 are activated by growth factors, serum, and phorbol esters and are involved in proliferation, differentiation and migration (Bardwell, 2006; Cuschieri and Maier, 2005; Murphy and Blenis, 2006; Nishimoto and Nishida, 2006). ERK1/2 can also be activated by environmental stresses, oxidative stress, LPS, and cytokines (Cuschieri and Maier, 2005; Nishimoto and Nishida, 2006; Roux and Blenis, 2004). Environmental stresses, oxidative stress, UV radiation, hypoxia, ischemia, LPS, and inflammatory cytokines activate the p38 isoforms (Cuschieri and Maier, 2005; Kaminska, 2005; Roux and Blenis, 2004). P38 MAPK are involved in inflammation, cell growth, cell differentiation, and cell death (Bardwell, 2006; Kaminska, 2005). JNK are activated by similar stimuli as p38 MAPK including cytokines, UV irradiation, LPS, and osmotic stress (Bardwell, 2006; Cuschieri and Maier, 2005; Kaminska, 2005; Roux and Blenis, 2004). The MAPKs are involved in the expression of inducible nitric oxide synthase in macrophages and ultimately, inflammation as well as a wide variety of cellular processes including activation of NADPH oxidase (Cuschieri and Maier, 2005; Dewas et al., 2000). The ERK1/2 inhibitor, PD98059, was able to inhibit the

phosphorylation of $p47_{phox}$ (Dewas et al., 2000). Paraquat induces apoptotic cell death through activation of the JNK signaling pathway in cultured neuronal cell line (Peng et al., 2005) and stimulates ERK1/2 to activate NADPH oxidase in microglia to generate more ROS in our data.

Nitric Oxide Synthase:

One possible source of oxidative stress is nitric oxide (NO) which is produced by nitric oxide synthase (NOS) by converting of L-agrinine to L-citrulline utilizing NADPH and O_2 as cofactors (Day et al., 1999; Duval et al., 1996; Ebadi and Sharma, 2003; Guo et al., 2001; Margolis et al., 2000). Three isoforms of NOS have been identified and they are classified as neuronal or type 1 NOS, immunologic or type 2 NOS, and endothelial or type 3 NOS (Day et al., 1999; Ebadi and Sharma, 2003). NOS1 and NOS3 are constitutive whereas NOS2 is inducible (Day et al., 1999; Shih et al., 2001). The constitutive NOS isoforms require calcium and calmodulin for activity and the inducible NOS requires de novo synthesis of the enzyme upon activation of transcriptional pathway by cytokines or LPS (Duval et al., 1996; Ebadi and Sharma, 2003; Noack et al., 1999). NOS2 plays a role in host defense and can produce a 1000-fold higher amounts of NO in the micromolar concentrations compared to the constitutive isoforms that only produces nanomolar NO concentrations (Guo et al., 2001; Yamamoto et al., 2007). NOS2 was first discovered in macrophages where it is involved in host defense, but NOS2 is also present in many other cells including certain types of neurons, astrocytes, and microglia (Connelly et al., 2005; Shih et al., 2001).

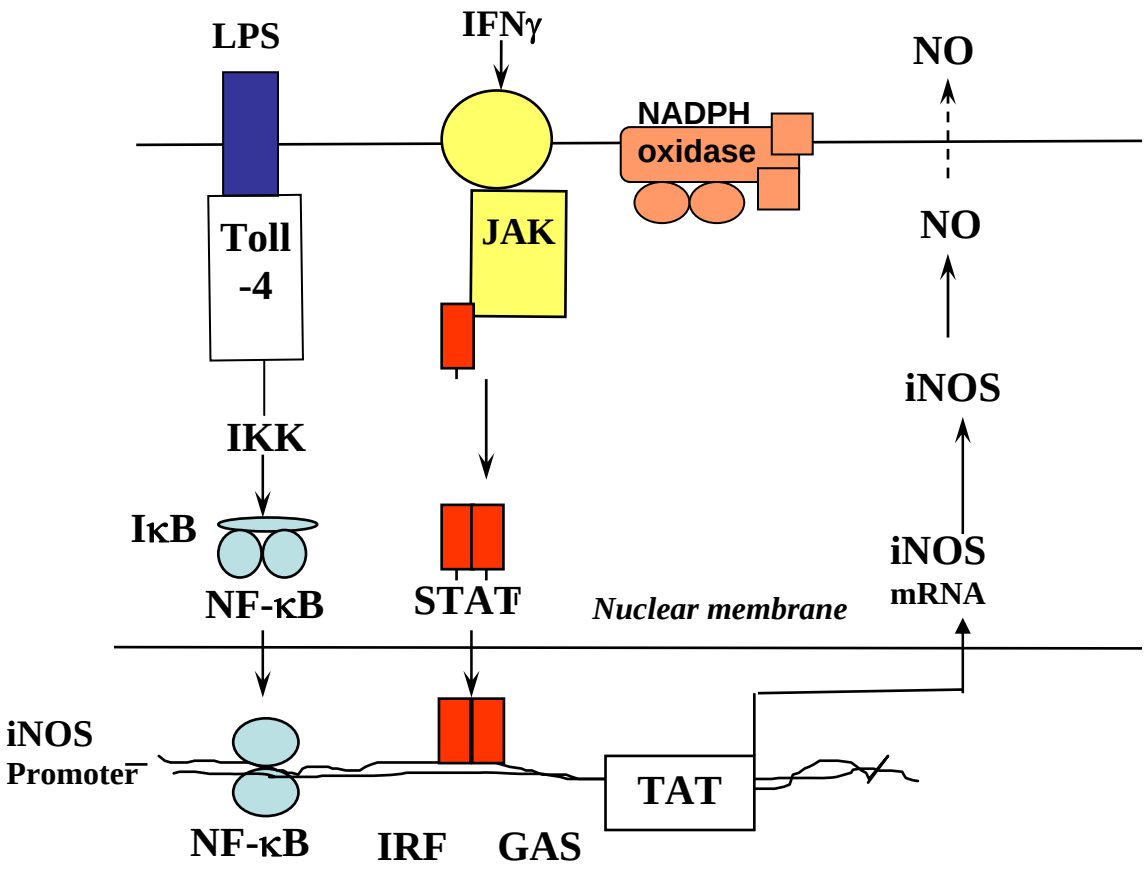

Fig 2. Schematic of the induction of inducible nitric oxide synthase by LPS and IFNγ

Unlike the other NOS which are constitutively express, iNOS mRNA and protein must be induced before the enzyme is active. Inflammatory molecules such as IFNγ and LPS can induce expression of iNOS. [This figure was adapted from an unpublished figure given by Dr. Grace Y. Sun in personal communication]

NO has diverse roles in the body depending on the concentration, the type of cell and the isoform expressed. It is regarded as a signaling molecule involved in a wide array of physiological events including smooth muscle relaxation, inflammation, host defense, cell death, blood pressure regulation, inhibition of platelet aggregation, atherosclerosis, and septic shock (Day et al., 1999; Duval et al., 1996; Guo et al., 2001; Mitsumoto and Nakagawa, 2001; Noack et al., 1999; Shih et al., 2001). In the brain, NO is involved in neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity, and gene expression (Day et al., 1999; Duval et al., 1996; Ebadi and Sharma, 2003; Guo et al., 2001; Xia et al., 1999). Because NO is a vasodilator, increased production may benefit the brain after injury (Gobbel et al., 1997). On the other hand, excess production of NO can cause neurodegeneration including stroke, PD and HIV dementia (Ebadi and Sharma, 2003; Tomita et al., 2001). NO can form adducts with mitochondrial enzymes and dysregulate mitochondrial function. There is a positive correlation between NO levels and cellular oxidation from the mitochondrial electron transport chain (Ebadi and Sharma, 2003; Guo et al., 2001; Yamamoto et al., 2007). Other possible mechanisms associating NO with neurodegeneration are inhibition of cytochrome oxidase, ribonucleotide reductase, superoxide dismutase, glyceraldehydes-3-phosphate, impairment of iron metabolism and generation of hydroxyl radicals and peroxynitrite (Ebadi and Sharma, 2003; Guo et al., 2001; Yamamoto et al., 2007).

Under many conditions, activated glia can simultaneously produce NO, ROS and cytokines and together, form the basis of many neuroinflammatory responses (Noack et al., 1999; Shin et al., 2001). Increased proinflammatory cytokines such as IL-1b and

interferon gamma (IFNγ) are observed in PD brains, and they can induce iNOS in activated microglia (Shen et al, 2004). Among the proinflammatory cytokines, IFNγ is produced by immune effector cells in response to intracellular pathogens (Gao et al., 2003c; Goralski and Renton, 2004; Iravani et al., 2005; Schroder et al., 2006; Whitehead et al., 2003). It is a pro-inflammatory cytokine that sensitizes immune cells to activation and rapidly alerts the cells of a possible infection (Schroder et al., 2006). Macrophages have a more rapid and heightened response to lipopolysaccharide (LPS) when first exposed to IFNγ (Schroder et al., 2006). The activated morphology of macrophages including cytoplasmic granules and pseudopodia is achieved after exposure to IFNγ and LPS (Schroder et al., 2006). In the murine immortalized microglial cells (BV-2), INFγ can induce iNOS and accelerate production of NO without other pathogens (Li et al., 1999; Shen et al., 2005).

Lipopolysaccharides:

Endotoxins such as LPS from bacteria are potent inflammatory molecules known to activate microglia and damage dopaminergic neurons (Arai et al., 2004; Herrera et al., 2005). LPS is a component of the outer membrane of gram-negative bacteria (Chandel et al., 2000; Goralski and Renton, 2004; West et al., 2000) and is a strong highly toxic stimulus for inflammatory responses in CNS (Goralski and Renton, 2004; West et al., 2000). LPS is more neurotoxic to dopaminergic neurons than neurons lacking tyrosine hydroxylase such as the gamma-aminobutyric acidergic and serotonergic neurons (Arai et al., 2004; Herrera et al., 2005). LPS can induce loss of dopaminergic neurons in substantia nigra (Gao et al., 2003c) and activate astrocytes and microglia to produce NO.

However, in the absence of glial cells, LPS is not neurotoxic to neurons, suggesting that glial cells mediate LPS neurotoxicity (Iravani et al., 2005). Activated microglia have been shown to be major mediators of the neurotoxicity of dopaminergic neurons by LPS (Arai et al., 2004). LPS failed to induce neurotoxicity in dopaminergic neurons when activation of microglial cells was inhibited (Li et al., 2005b). NOS2 inhibitors can also attenuate the loss of dopaminergic neurons induced by LPS, suggesting that NO is an important factor in mediating toxicity (Iravani et al., 2005). Pregnant rats treated with LPS gave birth to pups with fewer dopaminergic neurons than normal controls (Ling et al., 2004). The NOS2 gene has regulatory regions that have consensus elements for interferons and LPS (Duval et al., 1996).

Toxins that target dopaminergic neurons combine with LPS to synergistically enhance toxicity; toxicity was increased with MPTP and LPS treatment together or in tandem (Gao et al., 2003c). LPS alone did not induce dopaminergic neurotoxicity but enhanced the toxicity of MPTP (Goralski and Renton, 2004). MPTP and LPS induced additive generation of superoxide and NO and neurotoxicity was found only in the presence of microglia (Gao et al., 2003c). Apparently, MPTP and LPS induce ROS production through NADPH oxidase and activate inflammatory cytokine pathways for NO production (Ebadi and Sharma, 2003; Goralski and Renton, 2004). Inhibitors of NADPH oxidase and NOS2 can attenuate dopaminergic neurotoxicity due to MPTP and LPS (Gao et al., 2003c). These reactions require close proximity of neuron-glia; mixed cultures lacking functional NADPH oxidase were spared toxicity of MPTP and LPS (Gao et al., 2003c). Similarly, a combination of rotenone and LPS at nontoxic levels induced significant loss of dopaminergic neurons (Gao et al., 2003a). Prenatal exposure to LPS

lead to increased levels of oxidized proteins, and formation of Lewy body-like inclusions containing alpha-synuclein (Ling et al., 2004). Rotenone together with LPS further increased the number of activated microglia and synergistically caused the loss of dopaminergic neurons (Ling et al., 2004). Taken together, these results suggest that combinations of environmental toxins or drugs, even at nontoxic levels, may increase neuroinflammation and risk for development of PD (Goralski and Renton, 2004; Ling et al., 2004) .

Besides MPTP and rotenone, NO enhances the toxicity of paraquat. NO donors have been shown to exacerbate the toxicity of paraquat despite being minimally toxic by themselves (Gobbel et al., 1997; Tomita et al., 2001). Paraquat generates superoxide intracellularly in glial cells by redox cycling and increases susceptibility of cultures to produce NO (Gobbel et al., 1997; Noack et al., 1999). Pro-inflammatory cytokines may increase paraquat's toxicity through combined production of superoxide and NO (Tomita et al., 2001). In the presence of superoxide, NO can form peroxynitrite, a compound more toxic than either of the two agents (Gobbel et al., 1997). However, nitrotyrosine, an indicator of peroxynitrite, was not detected when an NO donor and paraquat were combined, suggesting that other factors are involved in the increased toxicity (Gobbel et al., 1997).

Toxins such as paraquat may cause toxicity by interacting directly with NOS; the underlying mechanism remains to be determined. Our results indicate that paraquat decreases NO generation of NO induced by IFNγ and LPS. Paraquat combined with cytokines significantly increased ROS generation, which disrupted membrane integrity more than paraquat alone. Similar to cytochrome P-450, NOS can be uncoupled by

shunting electrons away from the heme in the reductase domain to other molecules (Margolis et al., 2000). It is possible that in the presence of paraquat, electrons are shunted away from heme to paraquat which thus decreases NO production and increases superoxide production (Bonneh-Barkay et al., 2005; Margolis et al., 2000). When paraquat undergoes a one electron reduction, it produces a cation radical that can transfer its electron to oxygen to produce superoxide and paraquat (Bonneh-Barkay et al., 2005).

Conclusion

A number of models are available for PD but each has its strengths and weaknesses in mimicking the pathophysiological features of the disease. Models that mimic mitochondria dysfunction or amplify in oxidative stress are both supported by findings in postmortem studies of PD patients. There is also evidence that glia cells, especially microglia, play an active role in generation of ROS in PD. NADPH oxidase and NOS are known to play a role in inflammation and generation of ROS. Inhibition of these enzymes was protective to dopaminergic neurons. Activation of NADPH oxidase involves phosphorylation of the cytosolic subunits by protein kinases such as PKC and MAPK. These models suggest that besides the genetic component, environmental factors also play a role in the progression of PD. Furthermore, exposure of neurotoxins to neurons in substantia nigra can cause dopaminergic cell loss similar to PD. Future investigations to develop therapies for PD should focus on anti-oxidant and anti-inflammatory compounds that may inhibit ROS formation and suppress microglial activation.

CHAPTER 2

Cytotoxicity of paraquat in microglial cells: involvement of the PKC- and ERK 1/2-dependent NADPH oxidase

Cytotoxicity of paraquat in microglial cells: involvement of the PKC- and ERK 1/2-dependent NADPH oxidase

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Abstract:

Excess production of reactive oxygen species (ROS) is an important mechanism underlying the pathogenesis of a number of neurodegenerative diseases including Parkinson's disease (PD). PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra. Exposure to paraquat, an herbicide with structure similar to the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+), has been shown to produce PD-like symptoms. Despite previous focus on the dopaminergic neurons and signaling pathways involved in their cell death, recent studies have implicated microglial cells as the producers of ROS damaging neighboring neurons. In this study, we examined the source of ROS and the underlying signaling pathway for paraquat-induced cytotoxicity to BV-2 microglial cells. Paraquat-induced ROS production (including superoxide anion) in BV-2 cells was accompanied by translocation of $p67_{phox}$, the cytosolic subunit of NADPH oxidase, to the membrane. Paraquat-induced ROS production was inhibited by NADPH oxidase inhibitors, apocynin and diphenylene iodonium (DPI), but not the xanthine/xanthine oxidase inhibitor, allopurinol. Apocynin and DPI also rescued cells from paraquat-induced toxicity. While inhibitors for protein kinase C delta (PKCδ) or extracellular signal-regulated kinases (ERK1/2) could partially attenuate paraquat-induced ROS production and cell death, combination of these two kinase inhibitors completely abolished paraquat-induced cytotoxicity. Rottlerin, a selective PKCδ inhibitor, also inhibited paraquat-induced translocation of $p67_{phox}$. Taken together, this study demonstrates the involvement of ROS from NADPH oxidase in mediating paraquat cytotoxicity in BV-2 microglial cells and this process is mediated through the PKC- and ERK-dependent pathways.

Introduction:

Parkinson's disease (PD) is one of the most common neurodegenerative diseases in the elderly and is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (Schober, 2004). Although genetic mutations have been suggested for the juvenile onset of PD, for most cases, the cause is unclear and a number of environmental factors have been implicated in idiopathic PD (Firestone et al., 2005). Studies with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a toxin known to target dopaminergic neurons, demonstrated the involvement of oxidative stress in mediating the neuronal damage that resembled PD (Chun et al., 2001). Paraquat, an herbicide structurally similar to MPTP, has also been implicated in a variety of epidemiological studies (Peng et al., 2005). Similar to other dopaminergic toxins, paraquat has been shown to cause oxidative stress and selective death of dopaminergic neurons (Herrera et al., 2005; Peng et al., 2005).

Earlier studies have demonstrated a significant increase in markers for oxidative stress in the brains of PD patients as compared to controls (Beal, 2003; Koutsilieri et al., 2002; Shavali et al., 2006). In many instances, oxidative stress occurs before appearance of clinical manifestations (Koutsilieri et al., 2002). In studies with cellular models, paraquat was shown to increase oxidative stress and neurotoxicity in PC12 cells, and polyphenolic antioxidants could ameliorate the cytotoxicity (Koutsilieri et al., 2002; Yang and Sun, 1998a; Yang and Sun, 1998b). Aside from oxidative stress, studies with animal models have implicated microglia activation in the progression of PD (Croisier et al., 2005). Activated microglial cells were observed in primates given MPTP and humans accidentally exposed to MPTP (Barcia et al., 2004; Langston et al., 1999). However,

despite the increase in microglia in the PD brain as compared to normal controls, mechanism(s) linking activation of microglia to neuronal damage has not been elucidated.

Recent studies have provided strong evidence for microglia to produce ROS through activation of NADPH oxidase (Casarejos et al., 2006; Choi et al., 2005). This enzyme is comprised of multiple subunits in the cytosol and plasma membrane and assembly of the subunits is initiated by phosphorylation of the cytosolic subunits, e.g., $p47_{phox}$, $p67_{phox}$, $p40_{phox}$, and their translocation to the transmembrane subunits, e.g., $gp91_{phox}$, $p22_{phox}$ (Choi et al., 2005; Sumimoto et al., 2004). Paraquat has been shown to induce ROS in microglia through activation of NADPH oxidase (Bonneh-Barkay et al., 2005; Wu et al., 2005). However, mechanisms whereby paraquat stimulates ROS production and elicits cell damage remain unclear. Our preliminary data indicated the ability of paraquat to induce cytotoxicity on BV-2 microglial cells. In this study, we investigate whether ROS generated by NADPH oxidase may mediate the paraquat cytotoxicity in microglial cells.

Experimental procedures:

Cell Culture and Chemicals:

Microglia cells (BV-2) were obtained from R. Donato (University of Purugia, Italy) and were routinely cultured in 75 ml flasks containing DMEM (Gibco) with 5% FBS and 500 units/ml Penicillin/Streptomycin (Gibco). After confluent, cells were subcultured in 6 or 24-well plates for experiments. The chemicals used in the treatments include: U0126 (promega), GF109203x (Biosource), dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen), and dihydroethidium (DHE) (Invitrogen). The rest of the chemicals used are from Sigma: paraquat, rottlerin , 4-hydroxy-3-methoxy-acetophenone (apocynin), diphenylene iodonium (DPI), allopurinol, sodium diethyldithiocarbamate trihydrate (DDC), and 2',7'- 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Cells were routinely checked for contaminations and other morphological abnormalities prior to use in experiments.

Determination of ROS production:

For assessment of ROS produced by paraquat in the cell cytotoxicity experiment, the DCF-DA assay was used and cells were grown on 24 well plates until confluent. After removing serum, cells were placed in an isotonic Krebs-Ringer-Hepes (KRH) buffer containing 1.3 mM $CaCl_2$, 131 mM NaCl, 1.3 mM $MgSO_4$, 5 mM KCl, 0.4 mM KH_2PO_4, 6 mM glucose and 20 mM Hepes (pH 7.4). After equilibration for 2 h, DCF-DA (10 μg/ml) was added. After 1 hr the KRH buffer containing excess DCF was removed from the cells and replaced with fresh KRH and were treated by paraquat and inhibitors as described in individual experiments. Fluorescent intensity was measured at 485 nm

excitation and 520 nm emission on a Packard Fusion plate reader (Sheehan et al., 1997; Wang and Joseph, 1999). The KRH buffer was used in this assay because of high fluorescent background when using other medium e.g. DMEM. Testing the viability of cells with MTT assay comparing the KRH buffer to DMEM medium showed no significant difference in cell viability after 24 or 48 hours (data not shown).

DCF-DA is known to detect a number of ROS species, including superoxide, H_2O_2 and hydroxyl radical (Gomes et al., 2005). To better detect the superoxide anion, we also used the DHE protocol. DHE is oxidized to oxoethidium by superoxide anion and this highly fluorescent product binds to DNA (Miyata et al., 2005). Because the background fluorescence of DMEM:F12 did not interfere with the results in the DHE protocol, cells were grown on glass slides in serum-free and phenol red-free DMEM:F12 (1:1, by vol) and were incubated with 10μM DHE at 37°C for 30 minutes. After removing excess DHE and replacement with fresh phenol red-free DMEM:F12 medium, paraquat and/or inhibitor were added and cells were incubated at 37^{o}C for 4 hours. For quantitation of DHE fluorescence the glass slides were placed in an inverted fluorescent microscope (Nikon Eclipse TE2000-U) outfitted with a x 40 PlanFluor objective and a rhodamine filter set. Images were acquired using the MetaMorph software with a x 4 neutral density filter engaged. More than four separate images were taken per well. For determination of fluorescent intensity in individual cells, the MetaMorph software was used with proper background subtraction. Average intensity per cell was determined for more than 10 cells in each well and experiment was repeated three times.

Assessment of cell viability:

The MTT assay was used for determination of cell viability. Before treatment with paraquat and inhibitors, cells were placed in serum-free DMEM. Cells were incubated with paraquat and other agents for different periods up to 48 hours. After various treatments, culture medium was removed and 1 mg/ml MTT dissolved in DMEM was added to the cells and incubated for 2 hours. MTT was converted to blue formazan crystals by the dehydrogenases in active mitochondria in live cells. After incubation, medium was discarded, crystals dissolved in dimethyl sulfoxide:ethanol (1:1, by vol) and absorbance of the solution read at 560 nm (Li and Sun, 1999).

Immunoblot assay:

After incubation and treatment, cells were lysed with lysis buffer (150 mM NaCl, 50 mM tris pH 8, 2 mM EDTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS) plus protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) (Ruiz-Leon and Pascul, 2001). The lysate was then centrifuged for 15 minutes at 14,000 rpm. For isolation of membrane fractions, cells were suspended in a membrane separation buffer (0.25M sucrose, 5mM MgCl2, 2mM EGTA, 2mM EDTA, 10mM Tris (pH7.5), 10 μg/ml leupeptin, 1mM phenlymethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin) and centrifuged at 100,000g for 1 hour. The pellet was resuspended in membrane separation buffer with Triton-x 100 (Shmelzer et al., 2003). Proteins were separated on 10 % dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and transferred to nitrocellulose membranes at 200 mAmps. Membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST) for one hour at

room temperature. Primary antibodies were diluted in 2% milk in TBST. Primary antibodies include the following: polyclonal anti-$p67_{phox}$ 1:1000 (Upstate), monoclonal anti-β-actin 1: 2000 (Cytoskeleton), polyclonal anti-ERK1/2 1:1000 (cell signaling), and monoclonal anti-phospho-ERK 1/2 1:1000 (cell signaling). After incubation for 1 h at room temperature, excess primary antibody was washed off with TBST. Secondary antibodies, either anti-rabbit goat HRP conjugated (Santa Cruz) or anti-mouse goat HRP conjugated (Santa Cruz), were diluted in 2% milk in TBST. After incubation for 1 h at room temperature, excess secondary antibody was removed by washing with TBST. Bands were visualized with enhanced chemiluminescence (Pierce) and audioradiograph film (SciMart). Density of bands was measured using the Quantity One software.

Statistics:

All graphs were created using GraphPad Prism. Due to photo-bleaching and color and/or fluorescence contributed by some inhibitors that may interfere with the DCF assay, it was necessary to subtract fluorescent contributions by the inhibitors alone and normalize results to fold increase of control. Statistics were performed using One-way ANOVA with Newman-Keuls post test to compare all pairs of columns and a 95% confidence interval.

Results:

In this study, we first observed morphological changes induced by exposing BV-2 microglial cells to paraquat. In the presence of 5% FBS, these cells have a fast growing rate and mostly round shape with a halo around the rim. Cells deprived of serum for 24 or 48 hr do not show shape change as compared to those grown in serum (Fig. 1A and C). However, after addition of paraquat (50 μM) to these cells for 24 h, cells start to become condensed possibly an indication of apoptosis (Fig. 1B). After 48 h exposure to paraquat, many of the cells started to die and become detached (Fig. 1D). We further examined the time-course and dose-dependency for paraquat to induce cytotoxicity (using the MTT assay) and ROS production (using the DCF-DA assay). Paraquat exposure caused a dose- and time-dependent decrease in cell survival (Fig. 2A) and this effect was accompanied by the increase in ROS production (Fig. 2B). Exposure of microglia to paraquat (50 μM) significantly increased ROS production by 24 h (Fig. 2B). At this concentration, a significant cell death was not observed until 48 hours (Fig. 2A). Paraquat at 100 μM generated significant amounts of ROS at 24 h as well as detectable signs of cell death (Figs 2A and B). Because ROS production by paraquat appeared to plateau after 24 h, our subsequent studies used paraquat at 50 μM for 24 h for determination of ROS production and 48 h for assessment of cell survival.

In order to better determine the type of ROS produced by paraquat at the early onset, the dihydroethidium (DHE) method was used to detect whether it induced superoxide anion production in microglial cells exposed to 50 μM paraquat for just 4 h. As shown in Fig. 3B, paraquat (50 μM) did not increase DHE fluorescent intensity as

compared with control (Fig. 3A). However, an increase in DHE fluorescent intensity was observed upon treatment of microglia with diethyldithiocarbamate (DDC), a selective inhibitor of SOD, prior to paraquat exposure (Fig. 3D). In the presence of DDC, paraquat-induced cytotoxicity was evident after 4 h, probably due to accumulation of superoxide anions. In fact, treatment with DDC alone caused increase in DHE fluorescent intensity (Fig 3C) suggesting continuous production of superoxide in the cells under basal conditions.

Quantitative analysis of DHE fluorescent intensity of cells treated with paraquat and/or DDC are shown in Fig. 4A. In this experiment, we also compared ROS production as detected by the DCF-DA protocol under the same treatment conditions (Fig. 4B). This comparison indicated that although exposing cells to paraquat (50 μM) for 4 h did not cause a detectable increase in superoxide level (Fig 4A), a small but significant increase was observed when the DCF-DA protocol was used (Fig. 4B). DDC alone significantly increase the superoxide amounts, but it was not detected in DCF-DA. In addition, combination of DDC and paraquat significantly increased in fluorescent intensities in both DHE and DCF protocols with respect to the other groups (Fig. 4A and B). Therefore, paraquat induces superoxide anions, which are rapidly converted to H_2O_2 by SOD in the cells to detoxify them.

NADPH oxidase subunits are highly expressed in microglia cells (Jekabsone et al., 2006) and functional activity requires the assembly of cytosolic and membrane subunits. As a first step, we examined translocation of the cytosolic subunit, e.g., $p67_{phox}$, to the membrane upon exposure of microglial cells to paraquat. After treating cells with paraquat (50 μM) for 5 and 10 min, cells were harvested and fractionated to obtain the

cytosol and membranes for western blot analysis. There are high levels of $p67_{phox}$ in the cytosol; the ratios of $p67_{phox}$ to β-actin were not different (Fig. 5A). On the other hand, $p67_{phox}$ was significantly increased in the membrane fraction at 5 and 10 min after paraquat treatment (Fig. 5B and C).

In order to further test the involvement of NADPH oxidase in paraquat-mediated ROS production, inhibitors such as apocynin and diphenylene iodonium (DPI) were used. Apocynin is a methoxy-substituted catechol extracted from the root of *Picrorhiza kurroa* plant; it is a selective inhibitor of NADPH-induced ROS production as it blocks translocation of the cytosolic subunits to the membrane (Dodd-O and Pearse, 2000). DPI is not a selective inhibitor of NADPH oxidase as it can also inhibit electron transport through the cytochrome b (O'Donnell et al., 1993) as well as other redox cycling reactions. However, DPI has been extensively used as a supporting evidence for the presence of flavan catalyzed oxido/reduction reactions including NADPH oxidase (O'Donnell et al., 1994). As shown in Figs. 6A and B, apocynin inhibited paraquat-induced ROS production (detected after 24 h exposure) by 50% and rescued the cells from dying by 75 % (measured after 48 h). Pretreatment of cells with DPI (1 μM) almost completely abolished paraquat-induced ROS production (Fig. 6C). Due to interference of DPI with MTT, its effect on paraquat-induced cytotoxicity was not determined (O'Donnell et al., 1993). We also tested gp91ds-tat, a specific peptide inhibitor for NADPH oxidase, that was useful in other cells types in our lab (Krotz et al., 2007). However, for unknown reasons, this compound did not prevent paraquat-induced ROS production or cell death in microglial cells (data not shown). Possibly microglial cells do not readily take up this compound.

Xanthine oxidase is another enzyme known to generate superoxide and hydrogen peroxide (Biagi and Abate, 2005; Pacher et al., 2006). This enzyme is expressed widely in various organs including the brain (Pacher et al., 2006) and increased levels of it in the plasma and peripheral organs are indications of tissue damage (Biagi, 2005 and Abate; Pacher et al., 2006). In our study, we used two concentrations (10 and 50 μM) of allopurinol, a specific xanthine oxidase inhibitor, to evaluate effects on paraquat-induced production of ROS and cell death. As shown in Fig. 7, neither concentration was able to alter paraquat-induced ROS production and cell death.

PKC isoforms have been implicated in phosphorylation of the cytosolic subunits of NADPH oxidase (Grandvaux et al., 2001; Serezani et al., 2005; Waki et al., 2006; Zhao et al., 2005). In this study, we tested the possible involvement of PKC in paraquat-induced ROS production and cytotoxicity by treating microglial cells with GF109203x, a general PKC inhibitor with no selectivity for either conventional or novel PKC isoforms. As shown in Fig. 8A, GF109203x at 5 μM partially inhibited paraquat-induced ROS production by about 20% but ameliorated cell death by 60 % (Fig. 8B). When cells were treated with GF109203x alone, higher than 5μM caused cytotoxicity (data not shown). We further examined the effects of rottlerin, a selective inhibitor for PKCδ (Zhao et al., 2005). As shown in Figs. 8C, rottlerin (1 μM) partially inhibited ROS production and completely protected against paraquat-induced cell death (Fig 8D).

If PKCδ is essential for phosphorylating the NADPH oxidase subunits, it is reasonable that inhibition of PKCδ phosphorylation by rottlerin would prevent translocation of the cytosolic subunits. As shown in Fig. 9, paraquat-induced translocation of the $p67_{phox}$ subunit to membrane was completely inhibited by rottlerin,

providing support for paraquat-induced cytotoxicity through phosphorylation of the cytosolic subunits of NADPH oxidase by PKCδ.

Mitogen activated protein kinases (MAPKs) also have been implicated in the activation of NADPH oxidase subunits, particularly the $p47_{phox}$ subunit (Dewas et al., 2000; Karlsson et al., 2000). In order to test whether paraquat increases phosphorylation of ERK 1/2, we examined both p-ERK and total ERK in cells after paraquat treatment. As shown in Fig. 10A, treatment of microglial cells with paraquat caused the increase in phosphorylation of ERK1/2 within 5 and 10 min. The U0126, inhibitor for the MEK that phosphorylates ERK1/2, inhibited paraquat-induced ROS production by 50% and ameliorated cell death by 75% (Figs. 10B and C). Our results are in agreement with other studies showing ERK1/2 dependency in the activation of NADPH oxidase (Dewas et al., 2000; Karlsson et al., 2000).

The above studies demonstrated that PKC and ERK1/2 inhibitors could each partially reduce paraquat-induced ROS production and cytotoxicity. Because both kinases have been shown to phosphorylate NADPH oxidase subunits, this study was carried out to test if combination of both PKC and ERK1/2 inhibitors provides better protective effects than either alone. As shown in Fig. 11A and C, treatment of cells either with GF109203x and U0126 or rottlerin and U0126 significantly inhibited the production of ROS induced by paraquat. The combination of the inhibitors also attenuated paraquat-induced cell death (Fig 11B, D).

Discussion:

Microglia activation is known to play an important role in a number of neurodegenerative disorders including PD (Block et al., 2006; Li et al., 2005a; Li et al., 2005b; Qin et al., 2005; Zhang et al., 2005). Microglia are heterogeneously spread throughout the brain and are higher in the substantia nigra (12% of cells) than in the cortex (only 5%) (Kim et al., 2000; Lawson et al., 1990). In PD patients, the substantia nigra was found to have more than 6 fold the number of reactive microglia as compared to control brains (McGeer et al., 1988a; McGeer et al., 1988b). Microglia activation is involved in the cytotoxicity of neurotoxins such as MPTP, rotenone, substance P, and methamphetamine (Block et al., 2006; Delgado, 2003; Gao et al., 2002; Gao et al., 2003b; Scheller et al., 2005; Thomas et al., 2004; Wu et al., 2003). In the substantial nigra, a brain region enriched in dopaminergic neurons, microglial activation is associated with neurochemical changes such as the decrease in dopamine synthesis (Barcia et al., 2004; Scheller et al., 2005; Wu et al., 2003). In fact, changes in neurochemical parameters are observed before development of obvious lesions, suggesting that a short exposure to an insult may initiate a continuous process leading to neurodegeneration (Langston et al., 1999). Minocycline, an antibiotic shown to inhibit microglial cells, attenuated the neurotoxicity due to 6-hydroxydopamine, MPTP and rotenone (Casarejos et al., 2006; Croisier et al., 2005), further supporting the important role of microglia in the initiation and progression of PD. Using paraquat as a model compound, our study demonstrated time- and dose- effects for this compound to increase in ROS production and elicit cytotoxicity in microglial cells. In fact, paraquat-induced

cytotoxicity in microglial cells was more pronounced as compared to that in neuronal cells such as the SH-SY5Y cells (data not shown).

Recent studies have demonstrated NADPH oxidase as an important source of ROS produced by activated microglia (Li et al., 2005b) and ROS produced by glial cells can exert deleterious effects to neighboring cells including neurons (Abramov et al., 2004; Wilkinson, 2006). Neurotoxicity induced by substance P, rotenone and MPTP can be attenuated upon inhibition of NADPH oxidase by compounds such as DPI and apocynin or by using microglial cells deficient in NADPH oxidase (Block et al., 2006; Gao et al., 2002; Gao et al., 2003a; Wu et al., 2003). In agreement with these studies, paraquat-induced ROS production in BV-2 microglial cells inhibited by DPI and apocynin and these inhibitors also ameliorated paraquat-induced cell death. Although ROS can be produced by other oxidases including xanthine oxidase, negative results from testing with allopurinol further strongly suggest that xanthine oxidase is not a major contributor of the ROS in microglial cells.

Increases in ROS production by oxidases in the mitochondrial respiratory chain are regarded as important processes for toxin-induced apoptosis (Gonzalez-Polo et al., 2004; McCarthy et al., 2004). Mitochondrial dysfunction and mitochondrial-induced apoptosis have been demonstrated in paraquat toxicity in dopaminergic cells (Gomez et al., 2007; Gonzalez-Polo et al., 2004; McCarthy et al., 2004; Richardson et al., 2005). Our results show that paraquat-induced cytotoxicity (observed at 48 h) is preceded by the increase in ROS production (observed at 24 h). The total ROS produced during this latter period after exposure to paraquat includes the pool of ROS produced by mitochondria. Our results support the hypothesis that intricate mechanisms regulate the ROS production

in cells and in paraquat cytotoxicity, the pool of ROS produced by NADPH oxidase is an early event preceding mitochondrial ROS and cell death. Obviously, more studies are needed to further elucidate the link between these two pools of ROS. In a recent study with lung cells, paraquat was shown to cause a 2-4 fold increase in cellular oxygen consumption, which was neither due to NADPH oxidase nor mitochondrial respiration (Gray et al., 2007). This study further suggests the presence of a NAD(P)H:paraquat oxidoreductase in the lung cells for initiating the redox mechanism (Gray et al., 2007). Although our data demonstrated the involvement of NADPH oxidase in paraquat-induced ROS production, these results do not preclude the possibility of a similar oxidoreductase in microglial cells for initiating the redox reaction and activation of kinases that lead to translocation of the cytosolic subunits for assembly of NADPH oxidase.

Among more than 20 different PKC isoforms known to be present in mammalian cells, PKCδ is the most directly linked to activation of NADPH oxidase. There is evidence that overexpression of PKCδ increases generation of superoxide by NADPH oxidase (Talior et al., 2005) and inhibition of PKCδ, with rottlerin or expression of dominant negative PKCδ, attenuates the production of superoxide (Talior et al., 2005; Waki et al., 2006; Zhao et al., 2005). Inhibiting PKCδ with rottlerin or antisense oligodeoxyribonucleotides also inhibited phosphorylation of the $p67_{phox}$ and $p40_{phox}$ subunits (Bey et al., 2004; Grandvaux et al., 2001; Zhao et al., 2005). In our previous study using a special Western blot protocol to analyze PKC isoforms (Xu et al., 2002), we found that only 5 identifiable PKC isoforms are present in BV-2 microglial cells and that PKCδ is the most highly expressed among the isoforms (data not shown). The high abundance of PKCδ in BV-2 microglial cells clearly supports its role in kinase activity

and the effects of rottlerin in inhibiting paraquat-induced translocation of the $p67_{phox}$ subunit, ROS production and cell death. Future experiments using more molecular tools to verify site of action of PKCδ to phosphorylate specific NADPH oxidase subunit(s) will help in the development of specific pharmacological compounds targeting the signaling pathway for activation of NADPH oxidase.

MAPK activation, especially ERK1/2, has been shown in transgenic animal model of PD (Thiruchelvam et al., 2005). In our study, exposure of microglial cells to paraquat resulted in a rapid phosphorylation of ERK1/2 and inhibition of MEK by U0126 attenuated paraquat induced ROS production and cell death. Although antibodies for the $p47_{phox}$ subunit did not work in our hands for western analysis (data not shown), other studies have demonstrated ability for a MEK inhibitor to block phosphorylation of this subunit (Dewas et al., 2000; Karlsson et al., 2000). In agreement with results from a study by Dewas et al. (2000), our results show that inhibition of both PKC and ERK1/2 could completely abrogate the cytotoxic effects of paraquat. Taken together, these results are in agreement with the notion that multiple kinases are needed for phosphorylation of the NADPH oxidase subunits and assembly of the enzyme complex.

Among different NADPH oxidase inhibitors, the ability for apocynin to inhibit ROS production has generated special interest. This botanical compound is derived from the root of *Picrorhiza kurroa* (Dodd-O and Pearse, 2000) and has been used as a Chinese medicinal herb for treatment of a number of infectious and inflammatory diseases (Hougee et al., 2006; Kim-Mitsuyama et al., 2005; Lafeber et al., 1999). Studies from this lab also showed the ability for apocynin to protect against delayed neuronal death induced by global cerebral ischemia in the gerbil (Wang et al., 2006). Future studies with

PD animal models may provide information regarding whether apocynin can be a therapeutic compound for ameliorating PD-like symptoms induced by neurotoxins.

In summary, results from our study demonstrated cytotoxicity of paraquat to microglial cells through ROS produced from NADPH oxidase (Fig. 12). Our data also show that agents inhibiting NADPH oxidase not only inhibited ROS production but also rescued cells from cytotoxic effects of paraquat. Results from this study underline the importance of pharmacological agents targeting NADPH oxidase as therapeutic strategy for treatment and retardation of Parkinson's disease.

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Fig. 1. Representative photomicrographs depicting morphological changes in BV-2 microglial cells in serum free medium with or without paraquat (50 μM).

A: Control for 24h; B: paraquat treated for 24 h; C: control for 48 h; and D: paraquat treatment for 48 h. Arrows indicate morphology changes in paraquat treated cells. (Magnification: 200x)

Fig. 1

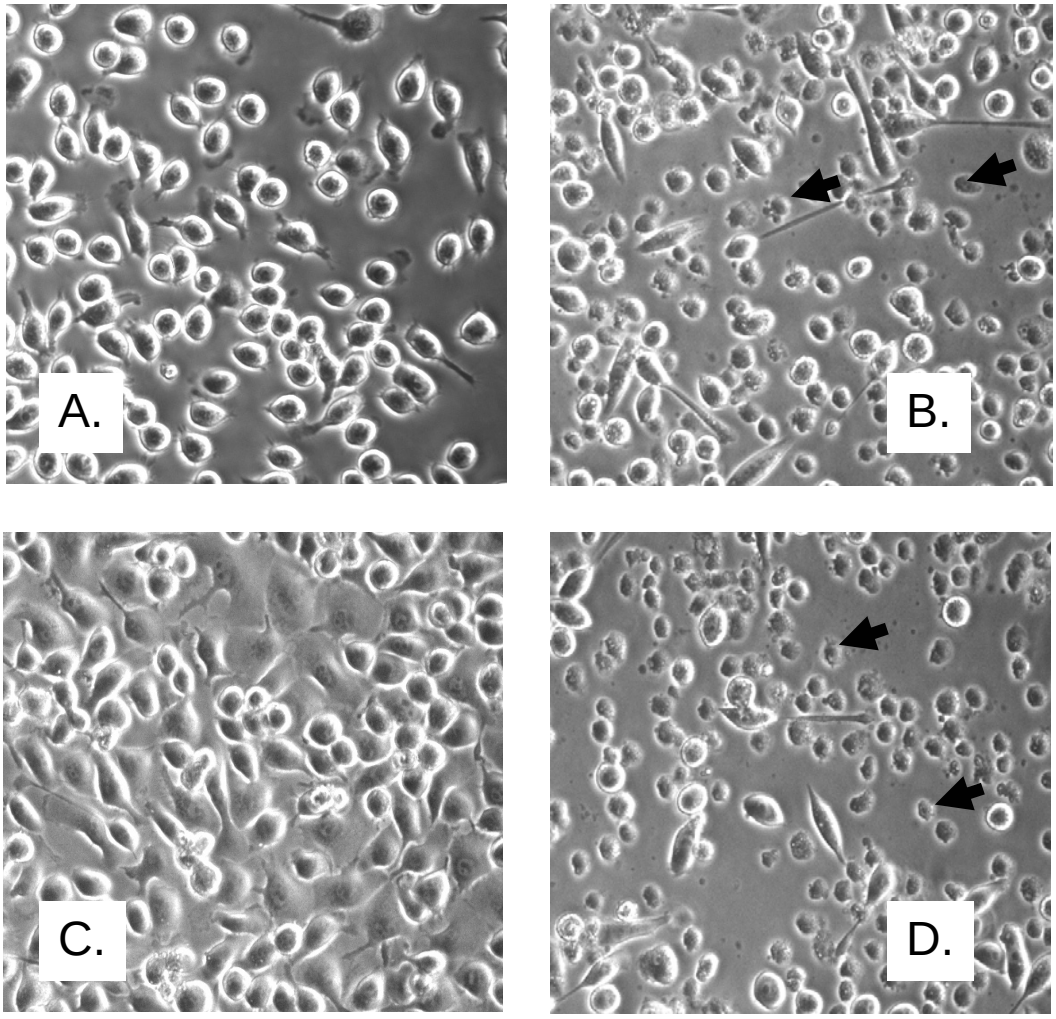


Fig. 2. Paraquat induced cytotoxicity and increased ROS production in BV-2 microglia cells.

A: Paraquat induced a time and dose-dependent decrease in cell viability as determined by MTT reduction assay as described in Method. B: Paraquat induced a time and dose-dependent increase in ROS production as determined by DCF-DA assay. Data are normalized to control and results are mean $\pm$ SD from 3 individual experiments.

Fig. 2

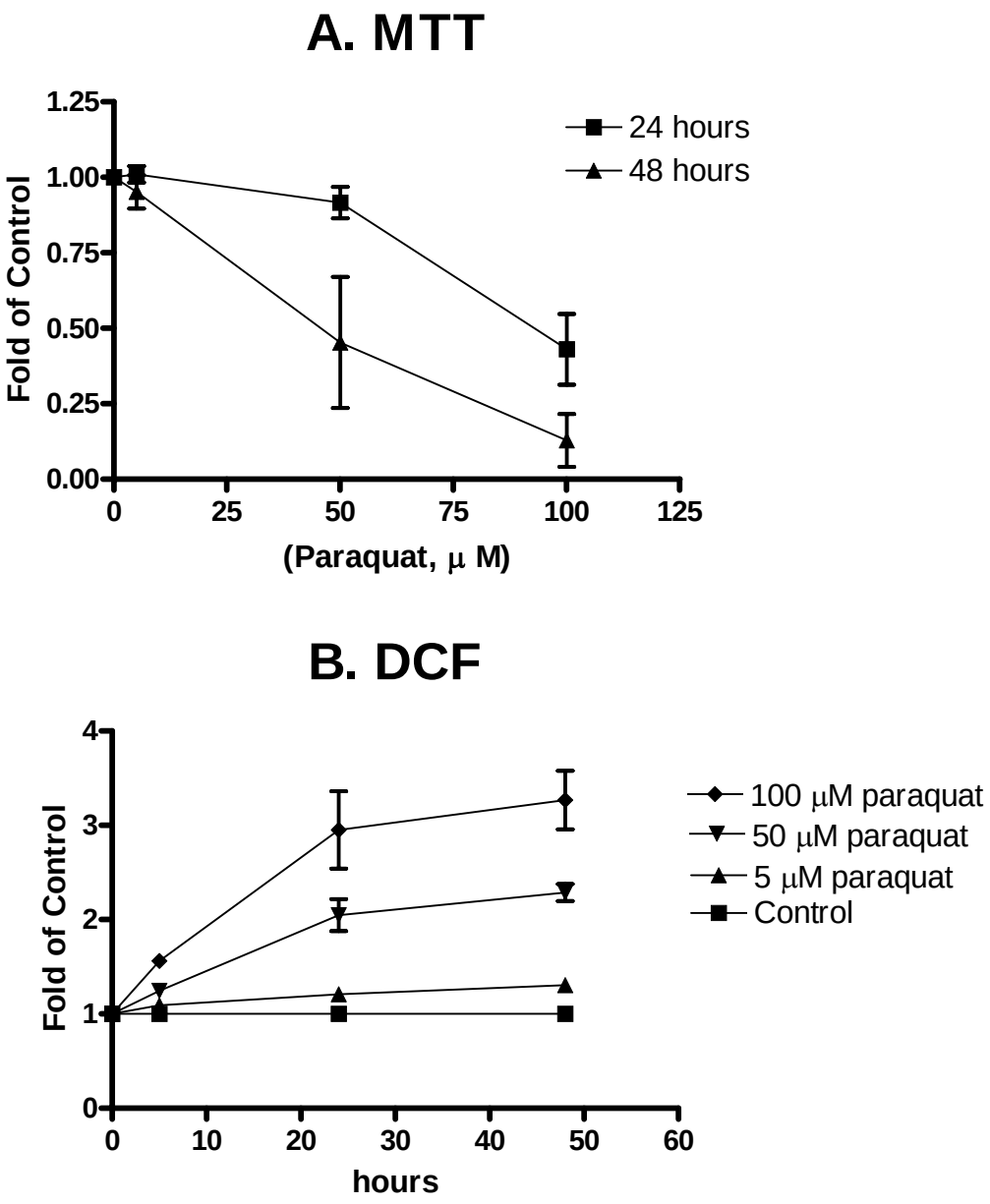

A. MTT
1.25
1.00
0.75
0.50
0.25
0.00
Fold of Control
0
25
50
75
100
125
(Paraquat, μ M)
24 hours
48 hours
B. DCF
4
3
2
1
0
Fold of Control
0
10
20
30
40
50
60
hours
100 μM paraquat
50 μM paraquat
5 μM paraquat
Control

Fig. 3. Paraquat caused the increase in superoxide anion as determined by the DHE assay protocol as described in Method.

Cells were treated with paraquat and/or DDC, a SOD inhibitor at 37^{o}C for 4 h. Results represent typical fluorescent micrographs depicting BV-2 microglial cells exposed to A: control; B: paraquat (50 μM); C: DDC (10 μM), and D: DDC and paraquat n=3.

Fig. 3

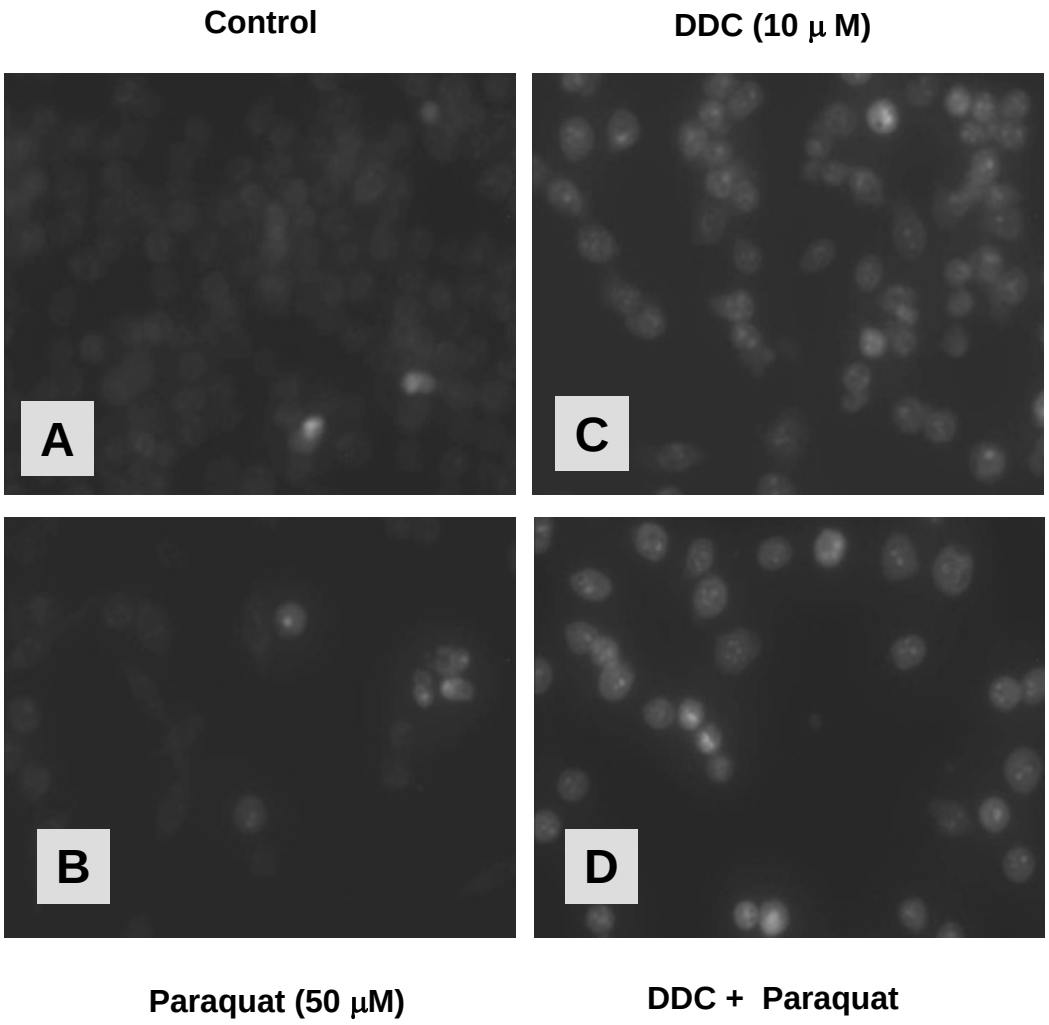


Fig. 4. Quantitative measurements of paraquat-induced ROS production by DHE and DCF in the presence and absence of DDC pretreatment.

The same experimental protocol as described in Fig. 2 for measurement of ROS using the DHE (A) and the DCF (B) protocols. Results are mean $\pm$ SD from three individual experiments. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control and [b] comparing DDC and paraquat with the other groups.

Fig. 4

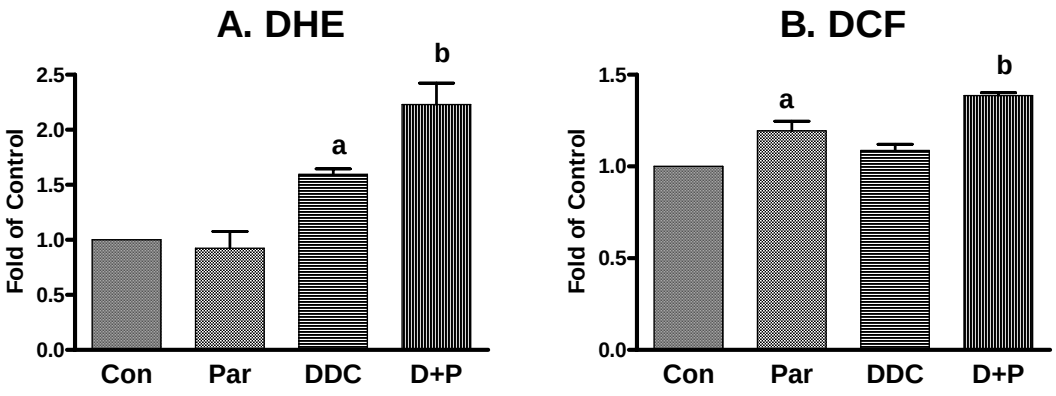

A. DHE
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
a
b
Con
Par
DDC
D+P
B. DCF
Fold of Control
1.5
1.0
0.5
0.0
a
b
Con
Par
DDC
D+P

Fig 5. A: Western blot analysis of p67 subunit of NADPH oxidase in cytosol and membrane fractions.

Cells were exposed to paraquat (50 μM) for 0, 5 and 10 min after which cells were subjected to fractionation to separate cytosol and membrane fractions as described in text. Beta-actin was used as loading controls. B: Density scan of the blots indicating ratios between $p67_{phox}$ and β-actin in membrane fraction from 3 independent experiments.[a]

[a] One-way ANOVA indicates significantly different from controls, $p < 0.05$.

Fig. 5

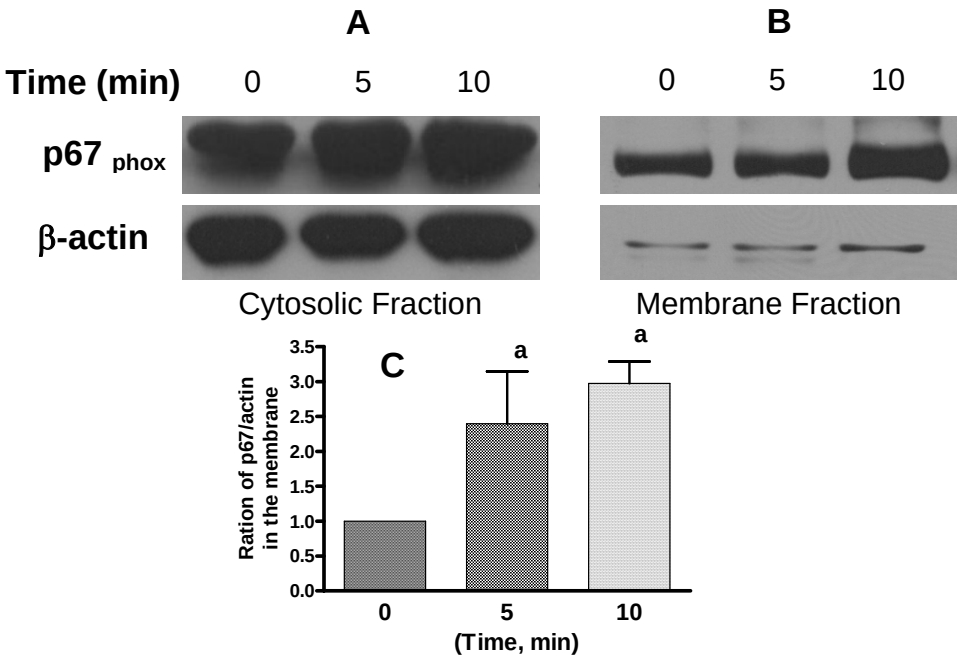

A
B
Time (min)
0
5
10
0
5
10
p67 phox
β-actin
Cytosolic Fraction
Membrane Fraction
C
a
a
Ration of p67/actin in the membrane
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
(Time, min)

Fig. 6. Effects of apocynin and DPI on paraquat-induced ROS production and cytotoxity.

Microglial cells were treated with paraquat (50 μM) and/or apocynin (1 mM) for (A) 24 h for ROS determination by DCF (n = 6), (B) 48 h for assessment of cytotoxicity by MTT (n = 3), and (C) treatment with paraquat (50 μM) and/or DPI (1 μM) for 24 h for ROS determination by DCF (n = 5). Results are mean ± SD from number of experiments as indicated. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 6

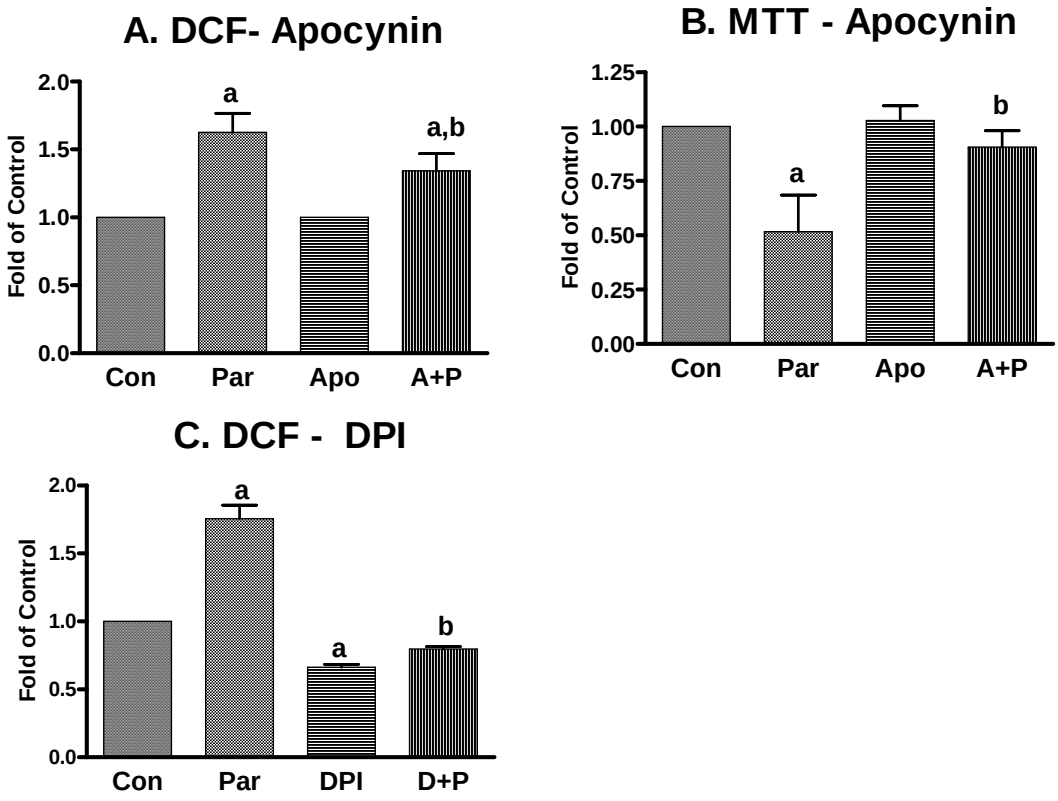

A. DCF- Apocynin
Fold of Control
2.0
1.5
1.0
0.5
0.0
a
a,b
Con
Par
Apo
A+P
B. MTT - Apocynin
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
Apo
A+P
C. DCF - DPI
Fold of Control
2.0
1.5
1.0
0.5
0.0
a
a
b
Con
Par
DPI
D+P

Fig. 7. Effects of xanthine oxidase inhibitor on paraquat-induced ROS production and cytotoxicity.

Cells were treated with paraquat (50 μM) and/or allopurinol (10 and 50 μM) for 24 h for (A) ROS determination by DCF and (B) for 48 h for assessment of cytotoxicity by MTT. Results are mean ± SD from 3 independent experiments. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control.

Fig. 7

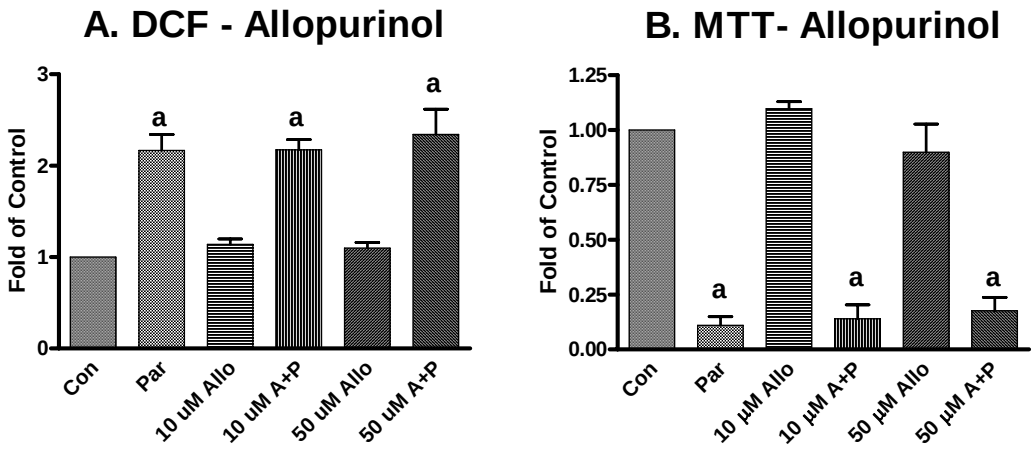

A. DCF - Allopurinol
Fold of Control
3
2
1
0
a
a
a
Con
Par
10 uM Allo
10 uM A+P
50 uM Allo
50 uM A+P
B. MTT- Allopurinol
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
a
a
Con
Par
10 µM Allo
10 µM A+P
50 µM Allo
50 µM A+P

Fig. 8. The role of PKC on paraquat-induced ROS production and cytotoxicity. Cells were treated with paraquat (50 μM), GF109203x (5 μM), and rottlerin (1 μM) for 24 or 48 h as described above. (A) Treatment with paraquat and/or GF109203x for 24 h for ROS determination; (B) treatment with paraquat and/or GF109203x for 48 h for assessment of cytotoxicity (C) treatment with paraquat and/or rottlerin for 24 h for ROS determination; and (D) treatment with paraquat and/or rottlerin for 48 h for cytotoxicity. Results are mean $\pm$ SD from 3-5 independent experiments. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 8

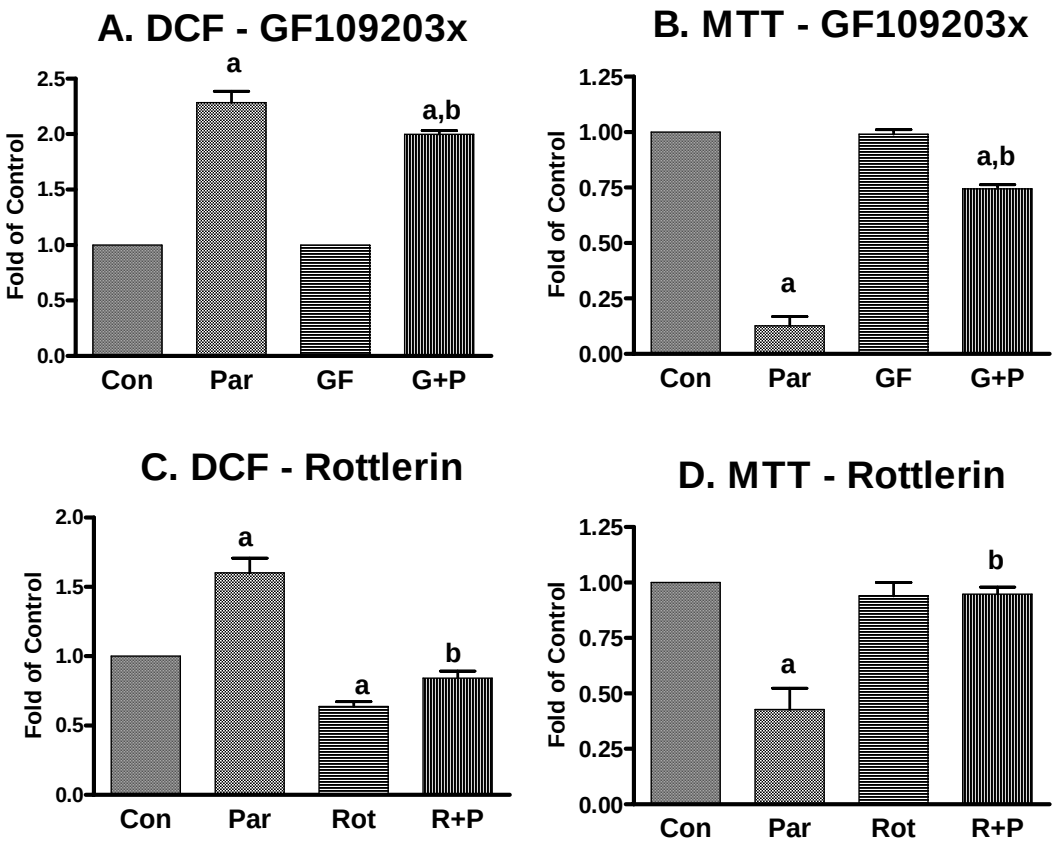

A. DCF - GF109203x
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
Con
Par
GF
G+P
B. MTT - GF109203x
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
a,b
Con
Par
GF
G+P
C. DCF - Rottlerin
Fold of Control
2.0
1.5
1.0
0.5
0.0
a
a
b
Con
Par
Rot
R+P
D. MTT - Rottlerin
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
Rot
R+P

Fig. 9. Effects of rottlerin on paraquat-induced translocation of p67 subunit to membranes.

Cells were exposed to paraquat and/or rottlerin for 24 h prior to separation of cytosol and membrane fractions for Western blot analysis. Blots were used for densitometer scanning and ratios of $p67_{phox}$ to β-actin in controls were normalized to 1. Results are expressed as mean $\pm$ SD (n = 4). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 9

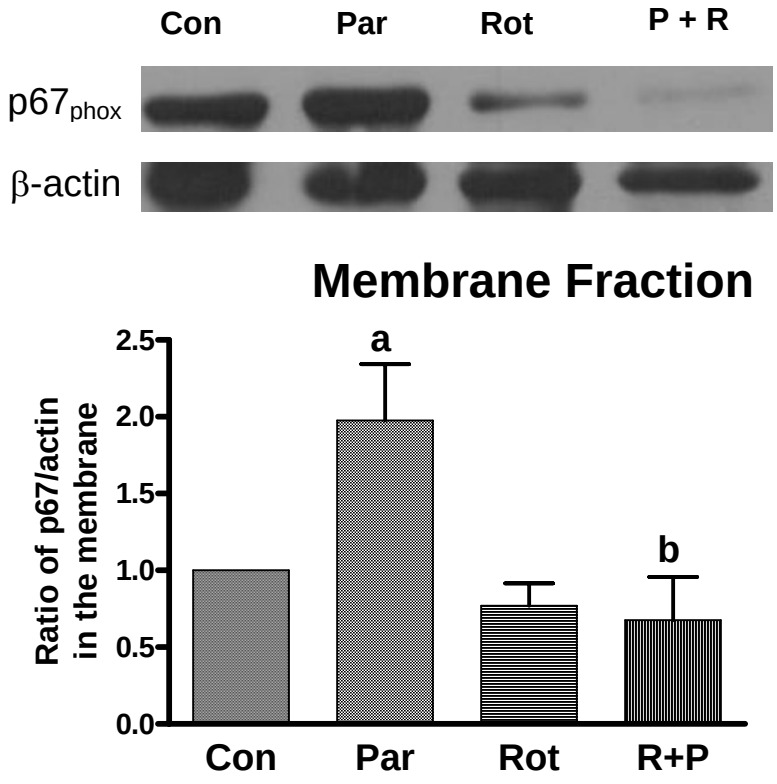
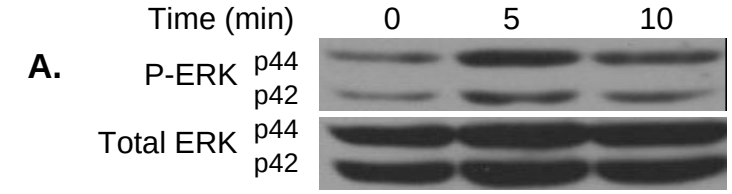
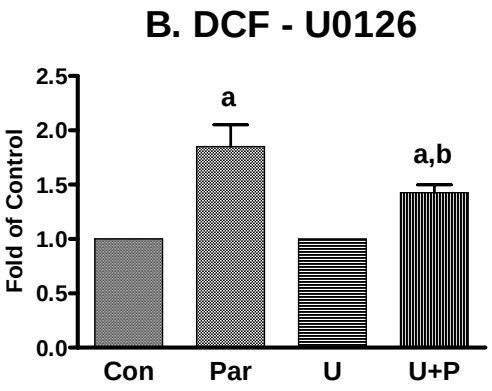

Con
Par
Rot
P + R
p67phox
β-actin
Membrane Fraction
Ratio of p67/actin in the membrane
2.5
2.0
1.5
1.0
0.5
0.0
a
b
Con
Par
Rot
R+P

Fig. 10. Paraquat induces phosphorylation of ERK1/2 and effects of MEK inhibitor on paraquat-induced ROS production and cytotoxicity.

(A) For demonstration of ERK activation, cells were exposed to paraquat (50 μM) for 0, 5 and 10 min prior to lysis for Western blot using antibodies for p-ERK and total ERK (n = 3). (B) Cells were exposed to paraquat (50 μM) and/or U0126 (10 μM) for 24 h for assessment of ROS (n = 5); (C) cells were exposed to paraquat (50 μM) and/or U0126 (10 μM) for 48 h for assessment of cytotoxicity (n = 3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 10


A.
Time (min)
0
5
10
P-ERK
p44
p42
Total ERK
p44
p42


B. DCF - U0126
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
Con
Par
U
U+P

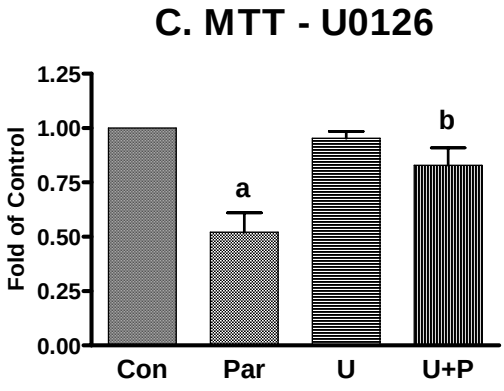

C. MTT - U0126
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
U
U+P

Fig. 11. Protective effects of PKC and MEK inhibitors on paraquat-induced cytotoxicity.

Cells were exposed to paraquat (50 μM) and/or GF109203x (5 μM) and U0126 (5 μM) (A) for 24 h for assessment of ROS generation (n=3) and (B) for 48 h for assessment of cytotoxicity (n = 3); cells were treated with paraquat and/or rottlerin (1 μM) and U0126 (5 μM) (C) for 24 h for assessment of ROS generation (n=3) and (D) for 48 h for cytotoxicity (n = 3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitors to paraquat.

Fig. 11

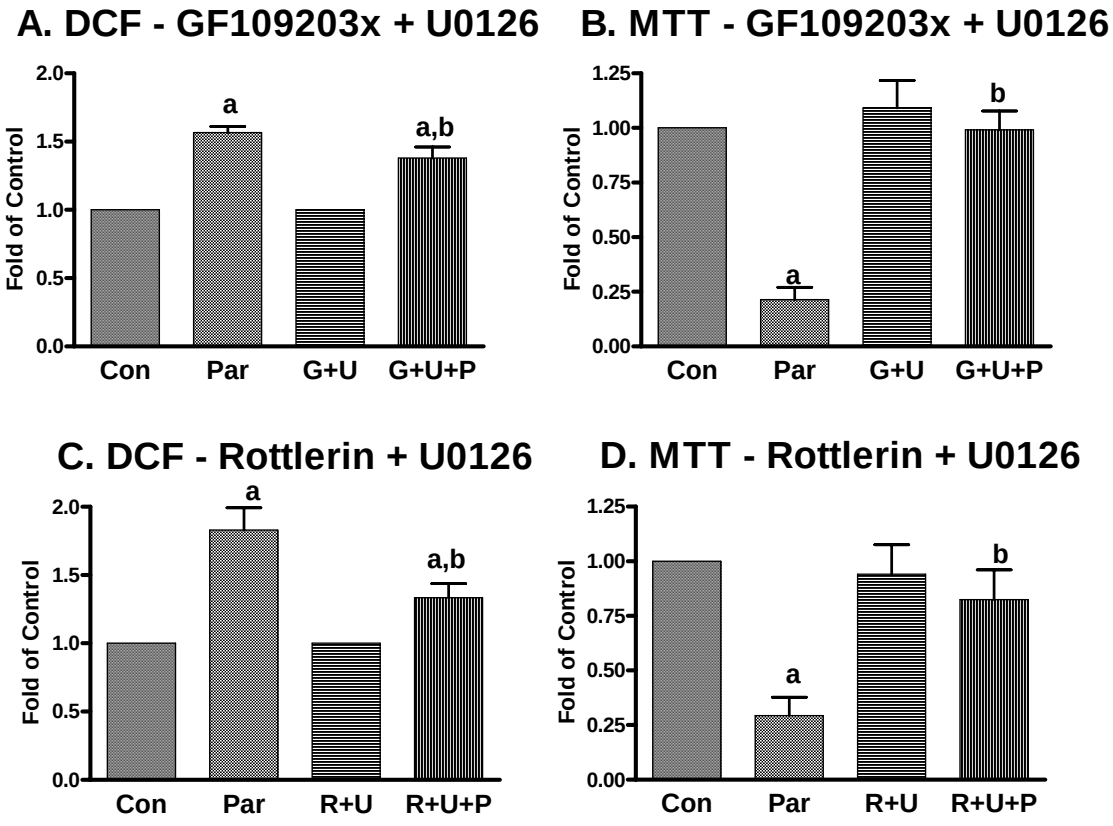
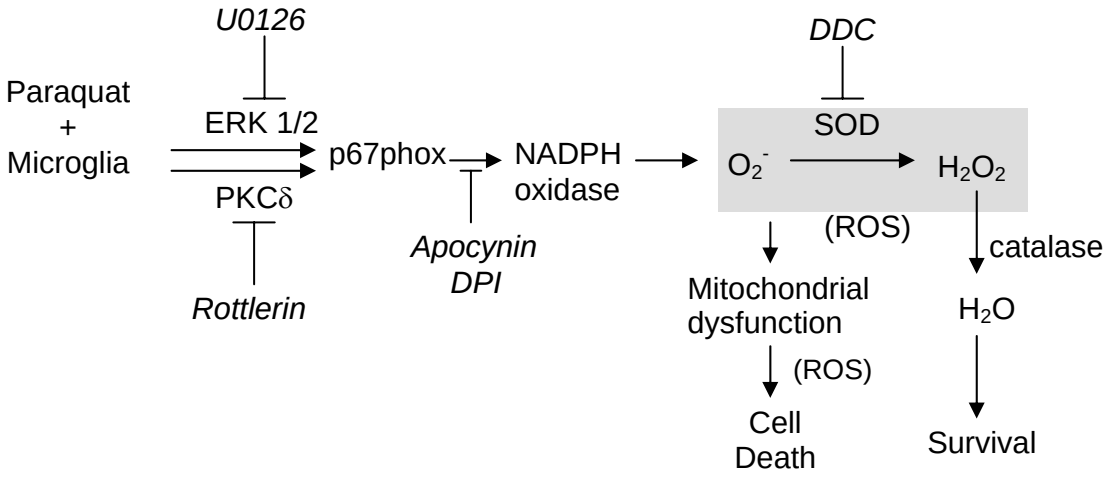

A. DCF - GF109203x + U0126
Fold of Control
2.0
1.5
1.0
0.5
0.0
a
a,b
Con
Par
G+U
G+U+P
B. MTT - GF109203x + U0126
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
G+U
G+U+P
C. DCF - Rottlerin + U0126
Fold of Control
2.0
1.5
1.0
0.5
0.0
a
a,b
Con
Par
R+U
R+U+P
D. MTT - Rottlerin + U0126
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
R+U
R+U+P

Fig. 12. A scheme depicting the signaling pathways for paraquat-induced ROS through activation of NADPH oxidase in microglial cells.

Fig. 12


U0126
Paraquat
+
Microglia
ERK 1/2
PKCδ
Rottlerin
p67phox
Apocynin
DPI
NADPH
oxidase
DDC
SOD
O2-
H2O2
(ROS)
catalase
Mitochondrial
dysfunction
H2O
(ROS)
Cell
Death
Survival

CHAPTER 3

The paradoxic effects of paraquat on BV-2 microglial cells: enhancement of ROS production and cytotoxicity but inhibition of cytokine-induced NO production

The paradoxic effects of paraquat on BV-2 microglial cells: enhancement of ROS production and cytotoxicity but inhibition of cytokine-induced NO production

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Abstract:

Reactive oxygen species (ROS) from microglial cells have been implicated in neurodegenerative diseases including Parkinson Disease (PD). Recent studies with paraquat, a dopaminergic neurotoxin, demonstrate the possible role of NADPH oxidase in production of superoxide in microglial cells. Under inflammatory conditions, microglial cells respond to interferon-γ (IFNγ) and lipopolysaccharides (LPS) in the induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO). Because paraquat can cause cytotoxicity through increasing ROS production in microglial cells, this study is to test if paraquat exacerbates cytotoxicity in the presence of NO produced by pro-inflammatory cytokines. Our results show that paraquat greatly attenuated cytokine-induced production of NO induced by both IFNγ and LPS but only moderately enhanced ROS production and cell cytotoxicity in microglial cells. Paraquat-induced attenuation of NO production was not due to inhibition of iNOS expression or a direct interaction between paraquat with nitrite in the culture medium. Paraquat treatment did not inhibit NO produced by S-Nitroso-N-acetyl-DL-penicillamine (SNAP), an NO donor. Inhibition of NO production by L-Nil, a specific inhibitor for iNOS, did not decrease ROS production by paraquat. Furthermore, paraquat did not increase levels of nitrotyrosine, a protein adduct due to peroxynitrite, in cells treated with IFNγ. On the other hand, a number of redox active compounds, including NADPH and menadione, were found to attenuate IFNγ-induced NO, suggesting that paraquat as well as these other compounds may inhibit cytokine induction of NO by interfering with the electron transport system for iNOS.

Introduction:

Parkinson's disease (PD) is a movement disorder that affects about 1% of the population over the age of 50 and is the second most common neurodegenerative disorder (Ebadi and Sharma, 2003; Shimizu et al., 2003). PD is characterized by a loss of dopaminergic neurons in substantia nigra resulting in dyskinesia, resting tremor, rigidity, and gait disturbance (Zhang et al., 2006). In over 90% of the cases of PD are non-familial, and although the cause for these cases remains elusive, oxidative stress has been regarded an important factor (Gao et al., 2003a; Shimizu et al., 2003). The brain is highly susceptible to oxidation due to high level of oxygen consumption, high proportions of polyunsaturated fatty acids, relatively low levels of endogenous antioxidants, physiological generation of reactive oxygen species, and elevated iron (Ebadi and Sharma, 2003). Dopaminergic neurons in particular are more susceptible to oxidative stress since they have lower content of antioxidants, higher accumulation of iron and metabolism of dopamine generates reactive oxygen species (ROS) (Gao et al., 2003b). Evidence of oxidative damage to lipids, proteins, and DNA and mitochondrial dysfunction has been shown in PD brain (Ebadi and Sharma, 2003).

Production of nitric oxide (NO) due to activation of microglial cells can cause oxidative stress. NO is generated by nitric oxide synthase (NOS) which converts L-agrinine to L-citrulline and requires NADPH and O_2 (Day et al., 1999; Duval et al., 1996; Ebadi and Sharma, 2003; Guo et al., 2001; Margolis et al., 2000). Three isoforms of NOS have identified that are classified as neuronal or type 1 NOS, immunologic or type 2 NOS, and endothelial or type 3 NOS (Day et al., 1999; Ebadi and Sharma, 2003). Type 1 and 3 NOS are constitutive and type 2 NOS is inducible (Day et al., 1999; Shih et al.,

2001). The constitutive isoforms require calcium and calmodulin to be active and the inducible isoform (iNOS) requires de novo synthesis of the enzyme upon stimulation by cytokines or LPS (Duval et al., 1996; Ebadi and Sharma, 2003; Noack et al., 1999). Cytokines such as IFNγ and lipopolysaccharide (LPS), component of the outer membrane of gram-negative bacteria, can independently stimulate iNOS in microglial cells (Chandel et al., 2000; Gao et al., 2003a; Goralski and Renton, 2004; West et al., 2000). Activation of microglia by LPS can induce loss of dopaminergic neurons in substantia nigra .

NO has diverse roles in the body depending on the concentration, the type of cells and the isoform expressed. In neurons, NO production is involved in neurotransmitter release, neurotransmitter reuptake, neurodevelopment, synaptic plasticity, and gene expression (Day et al., 1999; Duval et al., 1996; Ebadi and Sharma, 2003; Guo et al., 2001; Xia et al., 1999). Over production of NO has been shown to be involved in the neurotoxicity of several neurodegenerative disorders including stroke, PD and HIV dementia (Ebadi and Sharma, 2003). NO binds to the mitochondrial enzymes and decreases their function, and there is a positive correlation between NO levels and cellular oxidation from the mitochondrial electron transport chain . Other possible mechanisms that NO cause neurodegeneration are inhibition of cytochrome oxidase, inhibition of ribonucleotide reductase, impairment of iron metabolism, inhibition of superoxide dismutase, inhibition of glyceraldehydes-3-phosphate, and generation of peroxynitrite through interaction with superoxide (Ebadi and Sharma, 2003; Guo et al., 2001; Yamamoto et al., 2007).

Recent studies in our laboratory as well as by others show that paraquat is a redox active compound and can induce ROS in microglia through activation of NADPH oxidase (Bonneh-Barkay et al., 2005; Wu et al.., 2005). Because microglial cells also

respond to pro-inflammatory cytokines to produce NO, it is possible that superoxide produced by paraquat can combine with NO induced by cytokines to form peroxynitrite, a compound more toxic than either of the other two (Gobbel et al., 1997). In this study, we examined the interactive effects of cytokines and paraquat on NO production and cytotoxicity in microglial cells. In addition, we also examined the extent of peroxynitrite production under the oxidative and inflammatory conditions.

Experimental procedures:

Cell Culture and Chemicals:

Microglia cells (BV-2) were obtained from R. Donato (University of Purugia, Italy) and were routinely cultured in 75 ml flasks containing DMEM (Gibco) with 5% FBS and 500 units/ml Penicillin/Streptomycin (Gibco). After confluent, cells were subcultured in 6 or 24-well plates for experiments. The chemicals used in the study include: dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen), interferon γ (A.G. Scientific) and lipopolysaccharide (A.G. Scientific). Paraquat, NADPH, arginine, S-Nitroso-N-acetyl-DL-penicillamine (SNAP) and 4-hydroxy-3-methoxy-acetophenone (apocynin) were obtained from Sigma (St. Louis, MO). Cells were routinely checked for contaminations and other morphological abnormalities prior to use in experiments.

Determination of NO production:

To measure NO production the Greiss Reaction was used. Greiss Reaction actually measures nitrite and is incapable of detecting nitrate. Cells were cultured in 24 well plates until confluency. Prior to treatment, the growth medium was replaced with serum-free medium and treated with paraquat and cytokines as described in individual experiments. After 16 hours, 40 μL of the medium were removed to a 96 well plate. To each well, the following reagents (20 μL) were added : 7.5 mM sulfanilamide, 0.75 M HCl, and 7.5 mM 1-naphthyl ethylenediamine. Absorbance was measured at 450 nm on a Packard Fusion plate reader. A standard curve was also determined for each experiment. (Wang and Sun, 2001).

Determination of ROS production:

For assessment of ROS produced by paraquat in the cell cytotoxicity experiment, the DCF-DA assay was used. Cells were grown on 24 well plates until confluent. After removing serum medium, cells were placed in an isotonic Krebs-Ringer-Hepes (KRH) buffer containing 1.3 mM $CaCl_2$, 131 mM NaCl, 1.3 mM $MgSO_4$, 5 mM KCl, 0.4 mM KH_2PO_4, 6 mM glucose and 20 mM Hepes (pH 7.4). After equilibration for 2 h, DCF-DA (10 μg/ml) was added. After 1 hr the KRH buffer containing excess DCF was removed and cells were replaced with fresh KRH and were treated by paraquat and cytokines/inhibitors as described in individual experiments. Fluorescent intensity was measured at 485 nm excitation and 520 nm emission on a Packard Fusion plate reader (Sheehan et al., 1997; Wang and Joseph, 1999). The KRH buffer was used in this assay because of high fluorescent background when using other medium, e.g. DMEM. Testing the viability of cells with MTT assay comparing the KRH buffer to DMEM medium showed no significant difference in cell viability after 24 or 48 hours (data not shown).

Assessment of cell viability:

Cell viability was determined using LDH assay kit from Promega. Once cells in 24 well plates became confluent, the growth medium was replaced with serum-free medium and treated with paraquat and cytokines/inhibitors as described in individual experiments. After 24 hours, 50 μL of the medium were removed to a 96 well plate. The reconstituted substrate mix/assay buffer (50 μL) was added to each of well of 96 well plate and incubated for 30 minutes at room temperature protected from light. Stop

solution (50 μL) was added to each well. Absorbance was measured at 490 nm on a Packard Fusion plate reader (Riss and Moravec, 2004).

Immunoblot assay:

After incubation and treatment, cells were lysed with lysis buffer (150 mM NaCl, 50 mM tris pH 8, 2 mM EDTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS) plus protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) (Ruiz-Leon and Pascul, 2001). The lysate was then centrifuged for 15 minutes at 14,000 rpm. Proteins were separated on 10 % dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V and transferred to nitrocellulose membranes at 200 mAmps. Membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST) for one hour at room temperature or overnight at 4^{o}C. Primary antibodies were diluted in 2% milk in TBST. Primary antibodies include the following: polyclonal anti-iNOS 1:1000 (Upstate), and monoclonal anti-β-actin 1: 2000 (Cytoskeleton). After incubation for 1 h at room temperature or overnight at 4^{o}C, excess primary antibody was washed off with TBST. Secondary antibodies, either anti-rabbit goat HRP conjugated (Santa Cruz) or anti-mouse goat HRP conjugated (Santa Cruz), were diluted in 2% milk in TBST. After incubation for 1 h at room temperature, excess secondary antibody was removed by washing with TBST. Bands were visualized with enhanced chemiluminescence (Pierce) and audioradiograph film (SciMart). Density of bands was measured using the Quantity One software.

Assessment of Peroxynitrite:

Since peroxynitrite is short-lived in cells, nitrotyrosine, the product of peroxynitrite and tyrosine, was determined using the Nitrotyrosine ELISA assay kit from Cell Sciences (Canton, MA). Cells in six well plates were treated with paraquat (50 μM) and/or IFNγ (10 ng/ml) for 16 hours. Cells were lysed with lysis buffer and protein was normalized to 40 μg in each well. The assay was performed according to directions for the kit. Absorbance was measured at 490 nm on a Packard Fusion plate reader (ter Steege et al., 1998).

Statistics:

All graphs were created using GraphPad Prism. Due to photo-bleaching and color and/or fluorescence contributed by some inhibitors, which may interfere with the DCF assay, it was necessary to subtract fluorescent contribution by the inhibitors alone and normalize results to fold increase. Statistics were performed using One-way ANOVA with Newman-Keuls post-test to compare all pairs of columns and a 95% confidence interval.

Results:

In agreement with our previous study (Shen et al., 2005), LPS and IFNγ could individually cause NO production in BV-2 cells (Fig 1A, D). Exposing cells to paraquat did not cause NO production. Using DCF for determination of ROS, paraquat (50 μM) could enhance ROS production in microglial cells (Fig 1B, E). Under these conditions, LPS was not able to significantly increase ROS production, but IFNγ did significantly increase ROS production (Fig 1B, E). When microglial cells were exposed to LPS or IFNγ and paraquat, there were dramatic decreases in production of NO induced by LPS and IFNγ (Fig 1A, D). Paraquat dose-dependently decreased NO production and the detectable decrease could be observed at lower concentrations, e.g. 25 μM (data not shown). When cells were exposed to LPS or IFNγ together with paraquat (50 μM) for 16 h, ROS production was significantly ($p < 0.05$) greater than cells exposed to paraquat alone (Fig 1B, E). Furthermore, assessment of cell viability with LDH at 24 h after similar treatments indicated significant ($p < 0.05$) increases in cell death comparing paraquat together with LPS or IFNγ with paraquat alone (Fig 1C, F).

Because NO production from cytokine was measured in the form of nitrite in the culture medium using the Greiss reaction, a possible explanation for paraquat to inhibit cytokine-induced NO production is a direct reaction between paraquat and nitrite in the medium. In order to test this possibility, cells were exposed to LPS or IFNγ for 15 h, a time when maximum concentration of NO would have been produced, and paraquat was added to the culture for 1 h prior to removing the medium for NO measurement. As shown in Fig 2, paraquat added to the culture medium for 1 h prior to removing the

medium for measurement of NO did not attenuate the production of NO by LPS and IFNγ, suggesting paraquat does not directly oxidize nitrite in the medium.

Our recent study demonstrated the ability for paraquat to produce superoxide through coupling with NADPH oxidase (see Chapter 2). Since superoxide may interact with NO to produce peroxynitrite, the decrease in NO and increase in ROS due to addition of paraquat to IFNγ may be due to formation of peroxynitrite. The production of superoxide may account for the increase in ROS and the loss of membrane integrity. In order to determine if superoxide induced by paraquat was interacting with NO to generate peroxynitrite, we used SNAP, an NO donor, to replace iNOS induced by IFNγ or LPS. As shown in Fig 3A, paraquat was unable to decrease the NO produced by SNAP. Furthermore, there was also no significant difference between paraquat and paraquat and SNAP in the generation of ROS (Fig 3B). These results suggest that paraquat inhibition of NO is the result of a biochemical and not purely a chemical mechanism.

L-NIL is a selective iNOS inhibitor. Treatment of cells with L-NIL (500 μM) completely inhibited IFNγ induced NO production (Fig 4). Although L-NIL increased the basal generation of ROS in these cells, it also increased the generation of ROS in all groups by the same level (Fig 4B). Because DCF is a fluorescent measurement and the ROS in all groups are increase by the same amount, L-NIL itself possibly adds to the fluorescence and L-NIL did not have an effect on the ROS generation. Therefore, the data with L-NIL suggest that the concentration of NO in the cells was not the cause of increased ROS production due to IFNγ and paraquat (Fig 4).

The decrease in NO production could be due to inhibition of transcriptional synthesis of iNOS. Western blot analyses were carried out to examine iNOS protein in

microglial cells treated with paraquat and/or IFNγ. As shown in Fig 5, paraquat alone did not increase iNOS protein expression and paraquat together with IFNγ also did not alter iNOS expression as compared to that by IFNγ alone.

The formation of peroxynitrite from NO and superoxide could not explain the loss of NO in IFNγ and paraquat treatment. Since paraquat did not significantly decrease the production of NO, the superoxide generated by NADPH oxidase was not combining with NO to produce peroxynitrite (Fig 3). A protein adduct of peroxynitrite is nitrotyrosine. Paraquat and IFNγ did not significantly produce higher levels of nitrotyrosine as compared to that by IFNγ alone (Fig 7). Therefore, these data suggest that peroxynitrite formation is probably not the reason for paraquat to induce loss of NO.

The electron transport reaction for iNOS to convert arginine to citrulline and NO involves molecular oxygen and a number of cofactors, including NADPH and BH_4 (Day et al., 1999; Duval et al., 1996; Ebadi and Sharma, 2003; Guo et al., 2001; Margolis et al., 2000). We test the possibility that paraquat reduces NO production by interfering with the cofactors involved in the electron transport chain for iNOS. Because NADPH oxidase is a cofactor for both NADPH oxidase and iNOS, activation of NADPH oxidase induced by paraquat may decrease the availability of NADPH to iNOS. However, when exogenous NADPH was added to BV-2 cells, there was no further increase in ROS production by paraquat (data not shown) but instead, exogenous NADPH inhibited IFNγ-induced NO production (Fig 6). It is possible that extracelluar NADPH interferes with the Greiss reaction. Under the same conditions, addition of exogenous arginine, substrate for iNOS, there was no change in NO production by IFNγ in the presence and absence of paraquat (Fig 6).

Discussion:

Agents that induce the inflammation response such as endotoxin (LPS) have been shown to cause damage to dopaminergic neurons (Arai, 2004; Herrera, 2005). LPS is a component of the outer membrane of gram-negative bacteria and can elicit inflammatory responses in the CNS (Chandel et al., 2000; Goralski and Renton, 2004; West et al., 2000). LPS is highly toxic and strong stimuli for inflammatory responses in central nervous system (CNS) infections (Goralski and Renton, 2004; West et al., 2000). LPS is more neurotoxic to dopaminergic neurons than gamma-aminobutyric acidergic and serotonergic neurons (Arai, 2004; Herrera, 2005). LPS induces astrocytosis and microgliosis which is related to the expression of iNOS, but in the absence of glia cells LPS is not neurotoxic to neurons (Iravani et al., 2005). Microglial activation is regarded an important mediator of the neurotoxic response of dopaminergic neurons by LPS (Arai, 2004). LPS failed to induce neurotoxicity in dopaminergic neurons when microglia were inhibited (Li et al., 2005).

Interferon (IFN)γ is produced by immune effector cells in response to intracellular pathogens (Schroder et al., 2006; Whitehead et al., 2003). It is a pro-inflammatory cytokine that affects immune cells by sensitizing to activation and rapidly alerting them of a possible infection (Schroder et al., 2006). In many situations, macrophages show a more rapid and heightened response to LPS when first exposed to IFNγ (Schroder et al., 2006). Activated glial cells are associated with inflammation and produce NO, reactive oxygen species (ROS) and cytokines (Noack et al., 1999; Shin et al., 2001). Markers of

inflammation such as proinflammatory cytokine, IFNγ, and activated microglia have been detected in PD brains (Gao et al., 2003a; Goralski and Renton, 2004; Iravani et al., 2005).

In this study, we examined NO and ROS production in microglial cells in the presence of paraquat in combination of LPS or IFNγ. LPS and IFNγ can individually induce iNOS expression and produce NO in these cells (Shen et al., 2005). Studies in our laboratory (see chapter 2) as well as by others (Bonneh-Barkay et al., 2005) have demonstrated that paraquat produces ROS through NADPH oxidase. Results from this study clearly show that paraquat attenuated the generation of NO by LPS and IFNγ. Although the mechanism for the paraquat-induced decrease in NO is not clearly understood, there is increasing evidence that ROS and NO production from microglial cells are important underlying factors in mediating cytotoxicity to the dopaminergic neurons (Wu et al., 2005). The ability for paraquat to inhibit NO induced by both LPS and IFNγ suggests that the inhibitory effect is probably not due to action of paraquat on specific LPS or IFNγ receptors. We also eliminated the possibility that the decrease in NO is due to paraquat chemically reacts with NO (produced by NO donor) or nitrite in the medium. .

Expression of iNOS is associated with inflammation and is induced by cytokines and LPS (Duval et al., 1996; Ebadi and Sharma, 2003; Noack et al., 1999). iNOS inhibitors attenuated the loss of dopaminergic neurons induced by LPS suggesting that NO is involved in the toxicity of these neurons (Iravani et al., 2005). iNOS plays a role in host defense and can produce 1000-fold higher amounts of NO (in the micromolar concentrations) as compared to the other NOS isoforms that only produce nanomolar concentration of NO (Guo et al., 2001; Yamamoto et al., 2007). The possibility that

paraquat inhibits transcriptional pathways for induction of iNOS is also eliminated because paraquat did not decrease IFNγ-induced expression of iNOS.

Despite a dramatic decrease in NO, treatment of microglial cells with LPS and IFNγ together with paraquat resulted in a small increase in the production of ROS as compared with cells exposed to paraquat alone. Since the DCF assay is non-selective and also measures peroxynitrite, the increase in ROS may be due to interaction of NO with superoxide to form peroxynitrite (Gobbel et al., 1997). However, the lack of a significant increase in nitrotyrosine levels upon treating cells with paraquat and IFNγ as compared to that with IFNγ alone is surprising and argues against the possibility that all NO produced was converted to peroxynitrite under these conditions.

There is evidence that inflammation of the brain may increase the risk of developing PD when combined with environmental toxins or drugs (Goralski and Renton, 2004). Other neurotoxins that are known to target dopaminergic neurons combine with LPS to synergistically enhance neurotoxicity of the dopaminergic neurons (Gao et al., 2003a). LPS alone did not induce dopaminergic neurotoxicity but it could enhance the toxicity of MPTP (Goralski and Renton, 2004). Furthermore, the combination of MPTP and LPS was only neurotoxic in the presence of microglia (Gao et al., 2003b). In the study by Gao et al. (Gao et al., 2003a), MPTP and LPS additively increased generation of superoxide and NO. MPTP-induced activation inflammatory cytokine pathways in the brain and neurotoxicity of dopaminergic neurons could be inhibited by NOS inhibitor (Ebadi and Sharma, 2003; Goralski and Renton, 2004). Inhibitors of NADPH oxidase also inhibited the neurotoxicity of MPTP and LPS, and neuron-glia cultures lacking functional NADPH oxidase also prevented the toxicity of MPTP and LPS (Gao et al.,

2003b). Rotenone and LPS at nontoxic levels was shown to induce significant loss of dopaminergic neurons (Gao et al., 2003a).

NO donors exacerbate the toxicity of paraquat despite being minimally toxic by themselves (Gobbel et al., 1997; Tomita et al., 2001). Paraquat generates superoxide intracellularly by redox cycling and increases susceptibility of cultures to produce NO (Gobbel et al., 1997; Noack et al., 1999). Pro-inflammatory cytokines may increase the toxicity of paraquat through superoxide and NO (Tomita et al., 2001). The NO and superoxide can combine to form peroxynitrite, a compound more toxic than either compound alone (Gobbel et al., 1997). Our data and others show that nitrotyrosine, an indicator of peroxynitrite, was not detected when an NO donor and paraquat were combined (Gobbel et al., 1997). These data suggest that the increased toxicity due to paraquat and cytokines is probably through mechanism(s) other than peroxynitrite production.

We contemplate the possibility that paraquat or its oxidative products may interact directly with iNOS or its co-factors and thus decrease its activity. Similar to cytochrome P-450 (Margolis et al., 2000), conversion of arginine to citrulline by iNOS (also called NADPH diaphorase) involves NADPH, O_2, flavin adenine dinucleotide (FAD), flavin mononucleotide, heme and tetrahydrobiopterin (BH_4) (Moncada et al., 1997). The electron transport chain of cytochrome P-450 is uncoupled by paraquat (Margolis et al., 2000). Similarly, it has been shown that electrons are shunted away from the heme to paraquat with decreased of NO production and increased superoxide production (Bonneh-Barkay et al., 2005; Margolis et al., 2000). When paraquat undergoes a one electron reduction, it produces a cation radical and the electron can be

transferred to oxygen to produce superoxide (Bonneh-Barkay et al., 2005). In our study, we further demonstrated that besides paraquat, several other redox active compounds, including NADPH and menadione (data not shown), could decrease IFNγ-induced production of NO. These results lend strong suggestion that paraquat is uncoupling iNOS through redox cycling to produce superoxide instead of NO.

In conclusion, our results indicate that although paraquat attenuated the production of NO by IFNγ and LPS in microglial cells, these conditions increased ROS production and cell cytotoxicity. Although the mechanism for decreased NO production remains to be evaluated, there is evidence that besides production of peroxynitrite, paraquat may be physically interacting with the electron transport for iNOS to convert electrons normally used for NO production to superoxide production. The increase in generation of ROS and cell cytotoxicity induced by paraquat and cytokines suggest that neurotoxins exacerbate cytotoxicity under inflammatory conditions.

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Fig. 1. LPS and IFNγ effect on paraquat induced cytotoxicity and ROS production in BV-2 microglia cells.

Cells were treated with paraquat (50 μM) and/or LPS (10 ng/ml) A. generation of NO was measured at 16h with Greiss Assay (n=3), B. production of ROS as determined by DCF-DA assay after 16 h (n=3), and C: cytotoxicity determined by LDH assay (n=6). Cells were treated with paraquat (50 μM) and/or IFNγ (10 ng/ml) D. generation of NO was measured at 16h with Greiss Assay (n=4), E. production of ROS as determined by DCF-DA assay after 16 h (n=7), and F: cytotoxicity determined by LDH assay (n=3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control, [b] comparing LPS/IFNγ and paraquat with LPS/IFNγ and [c] comparing LPS/IFNγ and paraquat with the other groups.

Fig. 1

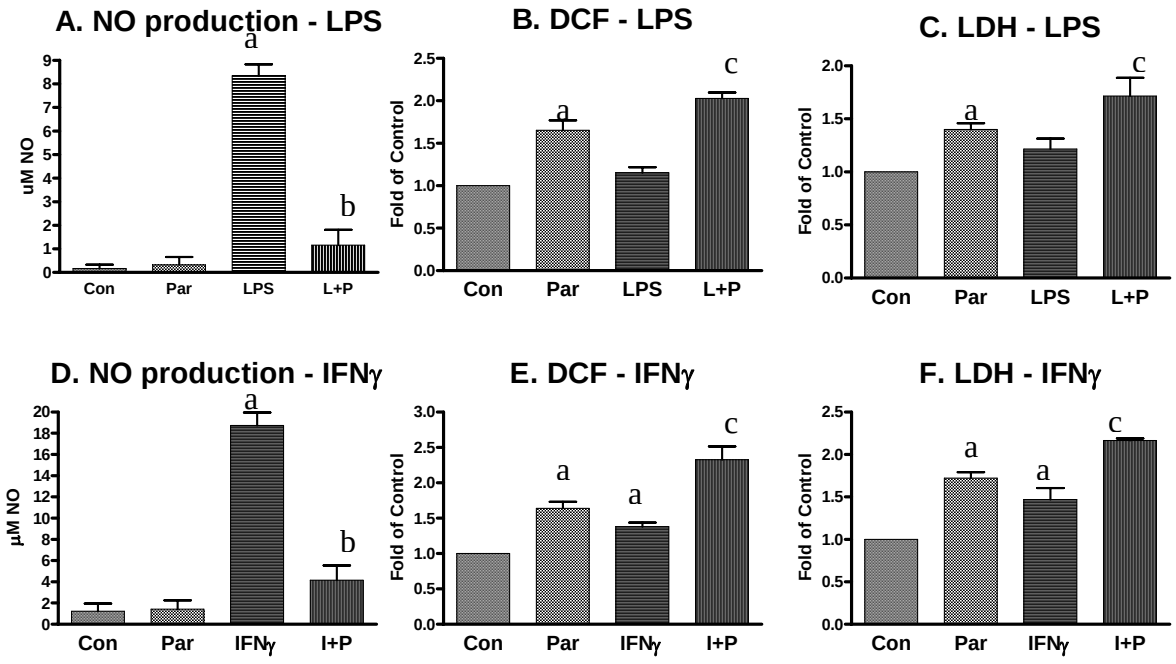

A. NO production - LPS
uM NO
Con
Par
LPS
L+P
a
b
B. DCF - LPS
Fold of Control
Con
Par
LPS
L+P
a
c
C. LDH - LPS
Fold of Control
Con
Par
LPS
L+P
a
c
D. NO production - IFNγ
µM NO
Con
Par
IFNγ
I+P
a
b
E. DCF - IFNγ
Fold of Control
Con
Par
IFNγ
I+P
a
a
c
F. LDH - IFNγ
Fold of Control
Con
Par
IFNγ
I+P
a
a
c

Fig. 2. Physical interaction with paraquat and NO.

Cells were treated with A.LPS (10 ng/ml) (n= 3)or B. IFNγ (10 ng/ml) (n= 3) for 15 h. Paraquat was added to the medium for 1h. NO was determined by Greiss Reaction. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control.

Fig. 2

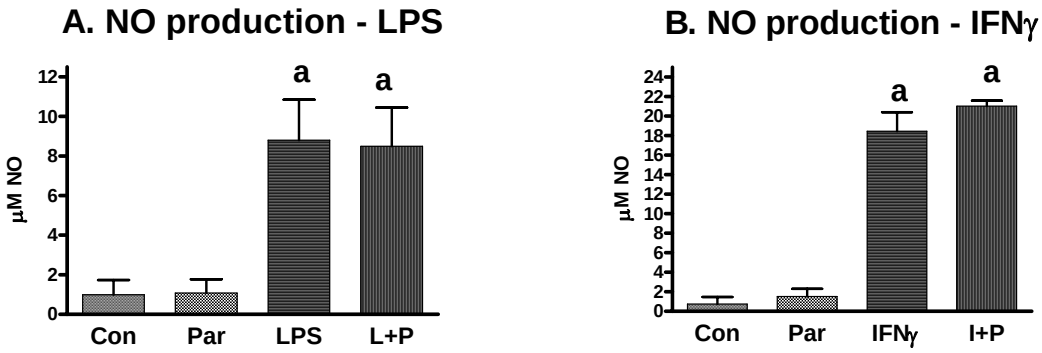

A. NO production - LPS
µM NO
12
10
8
6
4
2
0
a
a
Con
Par
LPS
L+P
B. NO production - IFNγ
µM NO
24
22
20
18
16
14
12
10
8
6
4
2
0
a
a
Con
Par
IFNγ
I+P

Fig. 3. The effect of SNAP, NO donor, and Paraquat on generation of NO and ROS.

Cells were treated with paraquat (50 μM) and/or SNAP (25 μM) for 16h. A. NO was determined by Greiss Reaction (n=3). B. ROS was measured by DCF-DA assay (n=3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control.

Fig. 3

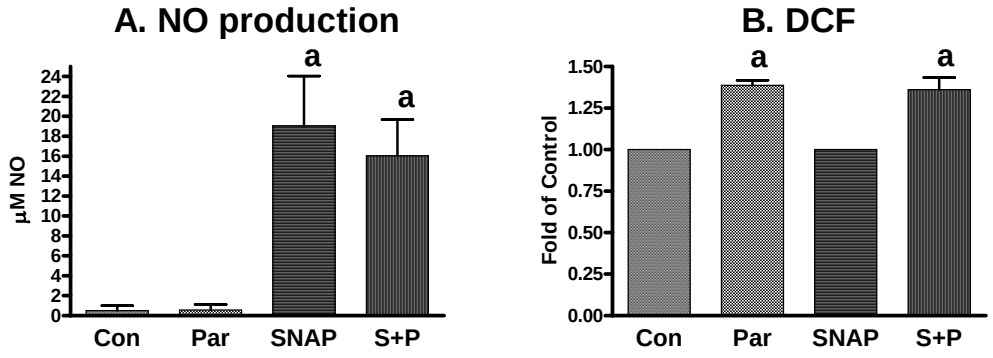

A. NO production
µM NO
24
22
20
18
16
14
12
10
8
6
4
2
0
a
a
Con
Par
SNAP
S+P
B. DCF
Fold of Control
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a
Con
Par
SNAP
S+P

Fig 4. L-NIL, iNOS inhibitor, effect on production of NO and ROS with paraquat and IFNγ.

Cells were treated with paraquat (50 μM) and/or IFNγ (10 ng/ml) and/or L-NIL (500 μM) for 16h. A. NO was determined by Greiss Reaction (n=3). B. ROS was measured by DCF-DA assay (n=3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control, [b] comparing treatment with IFNγ and [c] comparing L-NIL with treatment without L-NIL.

Fig. 4.

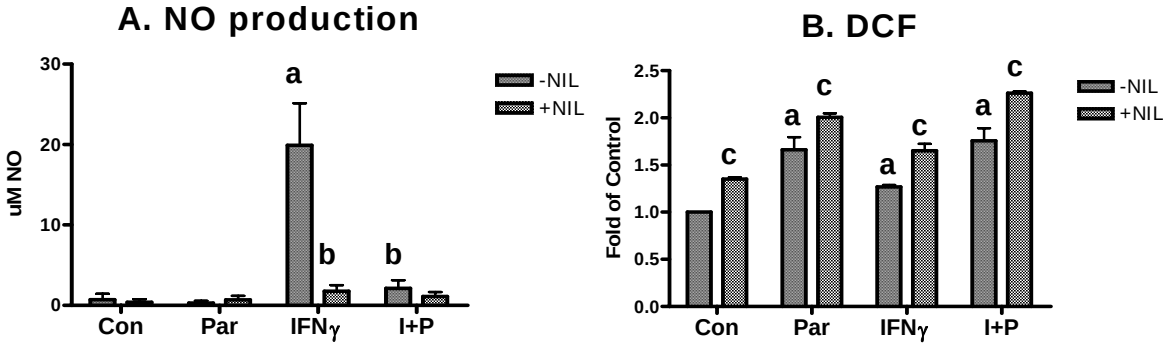

A. NO production
uM NO
30
20
10
0
a
b
b
Con
Par
IFNγ
I+P
-NIL
+NIL
B. DCF
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
c
a
c
a
c
a
c
Con
Par
IFNγ
I+P
-NIL
+NIL

Fig. 5. Expression of iNOS with IFNγ and paraquat.

Microglial cells were treated with paraquat (50 μM) and/or IFNγ (10 ng/ml) for 16 h. Cells were lysed and proteins separated by Western blot as described in methods. Blots were used for densitometer scanning and ratios of iNOS to β-actin of IFNγ were normalized to 1. Results are expressed as mean ± SD (n=5).

Fig. 5

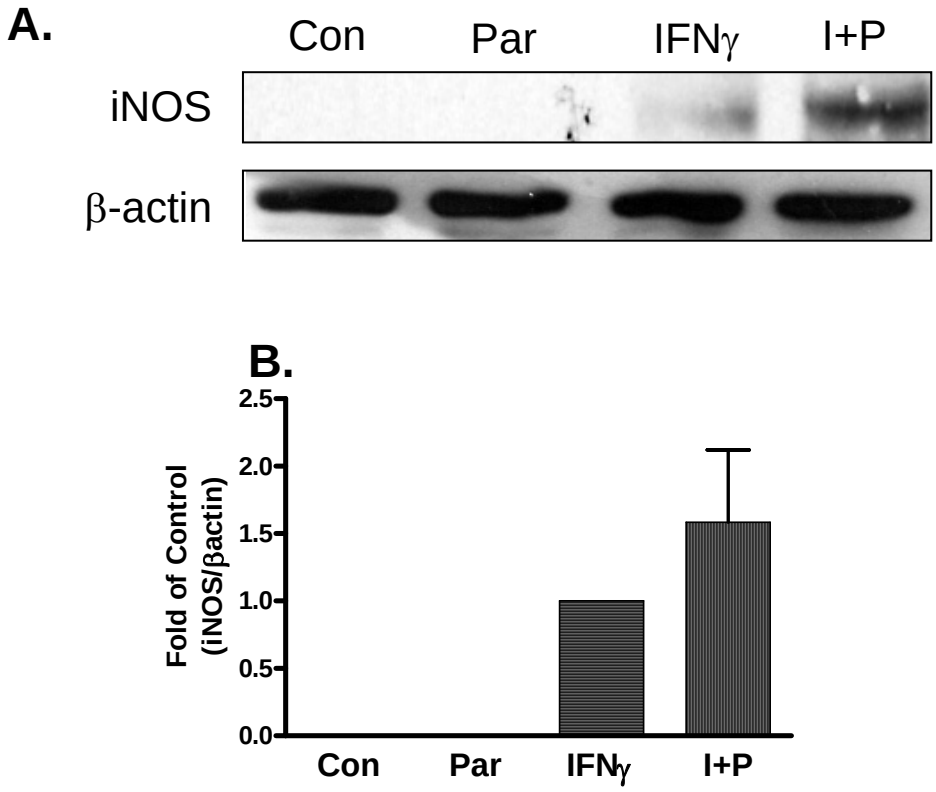

A.
Con
Par
IFNγ
I+P
iNOS
β-actin
B.
Fold of Control (iNOS/βactin)
2.5
2.0
1.5
1.0
0.5
0.0
Con
Par
IFNγ
I+P

Fig. 6. The effects of NADPH and arginine on paraquat inhibited IFNγ induced NO production.

A. Cells were treated with paraquat (50 μM), IFNγ (10 ng/ml) and NADPH (500 μM) for 16 h and NO was determined by Griess Reaction (n= 3). B. Cells were treated with paraquat (50 μM), IFNγ (10 ng/ml) and arginine (1 mM) for 16 h and NO was determined by Griess Reaction (n= 3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p< 0.05$: [a] comparing IFNγ with control and [b] comparing paraquat and IFNγ or IFNγ plus arginine or NADPH to paraquat and IFNγ or IFNγ.

Fig. 6

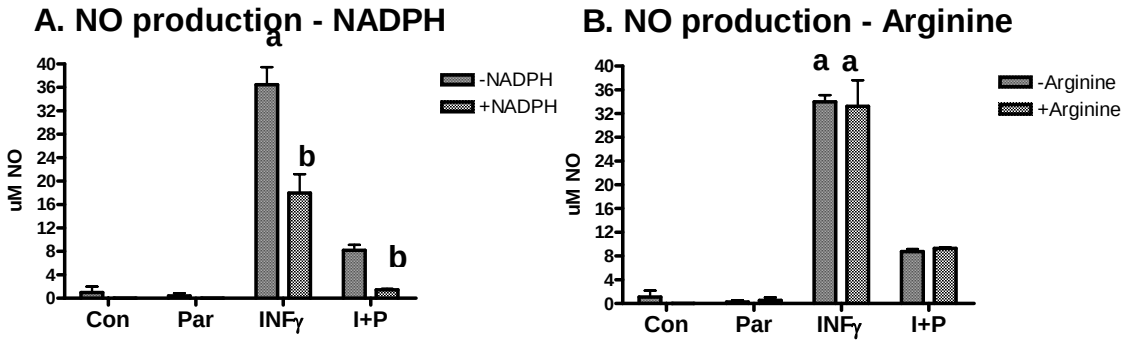

A. NO production - NADPH
uM NO
40
36
32
28
24
20
16
12
8
4
0
a
b
b
Con
Par
INFγ
I+P
-NADPH
+NADPH
B. NO production - Arginine
uM NO
a a
Con
Par
INFγ
I+P
-Arginine
+Arginine

Fig. 7. Effects of paraquat and IFNγ on nitrotyrosine production.

Cells were exposed to paraquat and/or IFNγ or SNAP for 16 h prior to lysing the cells for measurement of nitrotyrosine, a protein adduct of peroxynitrite, using the nitrotyrosine ELISA kit from Cell Sciences. A standard curve was used to determine the concentration of nitrotyrosine and correlated with peroxynitrite production. Results are expressed as mean $\pm$ SD (n = 3).

Fig. 7

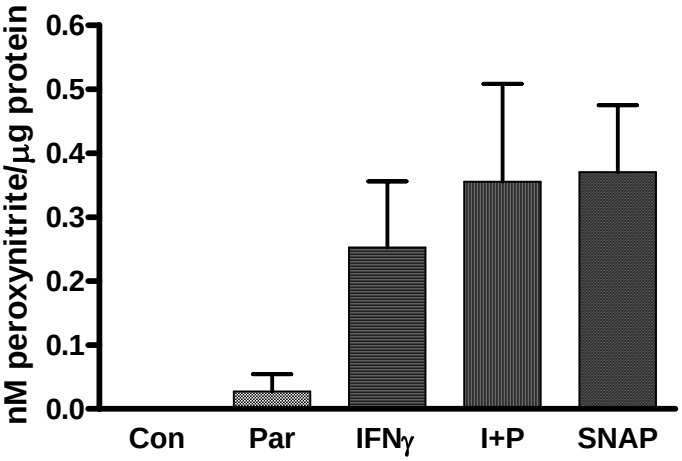
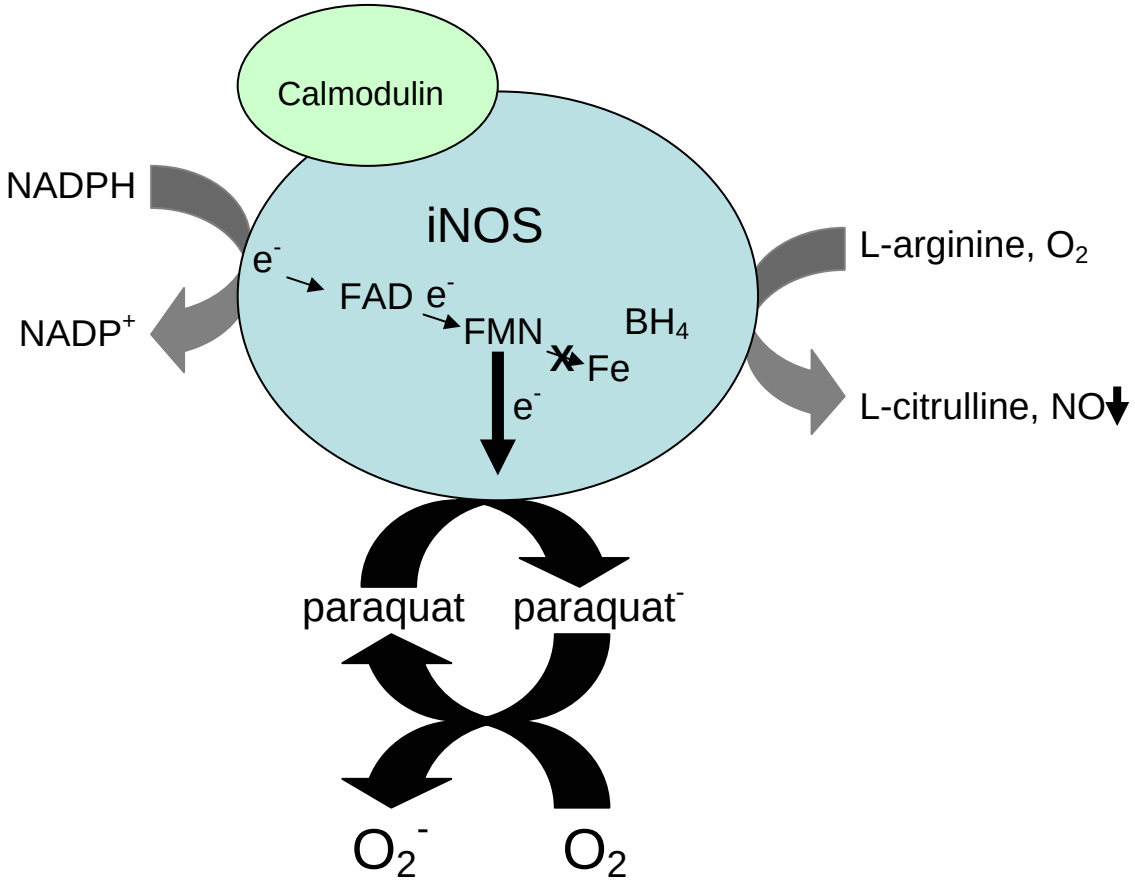

nM peroxynitrite/μg protein
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Con
Par
IFNγ
I+P
SNAP

Fig. 8. A scheme depicting paraquat-induced ROS through iNOS in microglial cells

Fig. 8


Calmodulin
NADPH
iNOS
L-arginine, O_2
e^-
FAD e^-
$NADP^+$
FMN
BH_4
Fe
e^-
L-citrulline, NO
paraquat
paraquat$^-$
O_2^-
O_2

CHAPTER 4

Paraquat induces upregulation of NADPH oxidase subunits in SH-SY5Y neuroblastoma cells

Parts of this chapter will be submitted to NeuroReport.

Paraquat induces upregulation of NADPH oxidase subunits in SH-SY5Y neuroblastoma cells

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Abstract:

Parkinson's disease (PD) is a neurodegenerative disorder known effect the dopaminergic neurons in the substantia nigra. Epidemiological studies have shown there is an increased risk of developing PD with exposure to paraquat. Paraquat is known to inhibit complex I of the mitochondrial respiration chain and induce generation of oxidative stress. Recently, the microglial NADPH oxidase has been implicated in the progression of PD. We wanted to determine the role of NADPH oxidase in neuronal cells. SH-SY5Y human neuroblastoma cells become dopaminergic neuronal-like after differentiation with retinoic acid. These differentiated cells express the NADPH oxidase subunit $p67_{phox}$ at lower amounts than do microglial cells. The NADPH oxidase inhibitor, gp91ds-tat, did not prevent paraquat toxicity. Paraquat did induce the translocation of $p67_{phox}$ to the membrane. Paraquat also induced the upregulation of the $p67_{phox}$ subunit protein and NOX2 mRNA. Increased expression of NADPH oxidase can significantly increase the production of superoxide. NADPH oxidase does not play a significant role in paraquat toxicity at high concentrations, but may play a role after prolonged and multiple low concentration exposures to paraquat similar to how people may be exposed to paraquat from the environment.

Introduction:

Over one million people in the United States are affected by Parkinson's disease (PD) (Chun et al., 2001). PD is a movement disorder in the elderly that generally effects people over the age of 55 (Schober, 2004; Siderowf and Stern, 2003). It is characterized by a progressive loss of dopaminergic neurons in the substantia nigra and presence of Lewy bodies or cytoplasmic protein deposits (Gao et al., 2003; Gelinas and Martinoli, 2002; Peng et al., 2005; Pesah et al., 2004; Thiruchelvam et al., 2000). Progressive loss of the dopaminergic neurons results in resting tremors, bradykinesia, abnormal postural reflexes, rigidity and akinesia (Pesah et al., 2004; Uversky, 2004). By the time the symptoms appear 50- 80% of the dopaminergic neurons have been lost (Schober, 2004; Uversky, 2004).

Epidemiological studies have identified several risk factors for PD including using well water, living on a farm, occupational use of herbicides, head trauma and family history of PD (Firestone et al., 2005; Semchuk et al., 1993). The majority of patients do not report a positive family history of PD, which suggests that factors other than genetic are involved (Semchuk et al., 1993). Over 90% of PD patients are non-familial cases with onset of the disease after the age of 50 (Chun et al., 2001; Gelinas and Martinoli, 2002; Shimizu et al., 2003b; Siderowf and Stern, 2003). Epidemiological studies have shown a positive correlation between increased risk for PD and exposure to herbicides containing paraquat (Brooks et al., 1999; Chun et al., 2001; Liou et al., 1997; Yang and Sun, 1998b).

Paraquat (PQ), 1,1'-dimethyl-4,4-bipyridinium, a widely used herbicide in the 20^{th} century (Brooks et al., 1999; Wesseling et al., 2001; Yang and Sun, 1998b). PQ is used to control weeds and grasses in agriculture fields (Shimizu et al., 2003a; Thiruchelvam et al., 2000; Yang and Tiffany-Castiglioni, 2005). There is an overlap between the geographic areas that paraquat is used and incidences of PD (Shimizu et al., 2003a). Paraquat is structurally similar to the active metabolite 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridium ion (MPP^+) that is known to induce parkinsonism (Brooks et al., 1999; Chun et al., 2001; Peng et al., 2005; Shimizu et al., 2003a; Thiruchelvam et al., 2000; Yang and Sun, 1998b). Paraquat decreased dopaminergic neurons in substantia nigra and caused the symptoms of Parkinsonism (Brooks et al., 1999; McCormack et al., 2002; Thiruchelvam et al., 2000; Wesseling et al., 2001; Yang and Sun, 1998a). Paraquat is selectively taken up in dopaminergic neurons through the dopamine transporter (Shimizu et al., 2003a; Yang and Tiffany-Castiglioni, 2005). In neuronal cells, paraquat inhibits the mitochondrial complex I of the mitochondrial respiration chain (Bretaud et al., 2004; Shimizu et al., 2003a; Yang and Tiffany-Castiglioni, 2005). This causes impaired energy metabolism, proteasomal dysfunction and intracellular reactive oxygen species that lead to malondialdehyde, protein carbonyls, and DNA fragmentation (Bretaud et al., 2004; Peng et al., 2005; Shimizu et al., 2003a; Thiruchelvam et al., 2005; Yang and Tiffany-Castiglioni, 2005).

Most of the research has focused on mitochondria dysfunction in neurons, but recent studies have suggest overactivity of the microglial NADPH oxidase involvement in PD. NADPH oxidase is an enzyme made up of two membrane components, NOX and $p22_{phox}$, and three cytosolic components, $p47_{phox}$, $p67_{phox}$, and $p40_{phox}$ (Bedard and

Krause, 2007; Geiszt, 2006). Recently, it has been found that NADPH oxidase is also in non-phagocytic cells including neurons (Zekry et al., 2003). Some non-phagocytic functions for NADPH oxidase include neuronal signaling, memory and cardiovascular homeostasis (Infanger et al., 2006). Dysregulation of NADPH oxidase is involved in neurotoxicity, neurodegeneration and cardiovascular diseases (Infanger et al., 2006). Seven isoforms of the NOX family had been described, including: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). The NOXs are enzymatic family that reduces oxygen to superoxide by transporting electrons across the membrane (Bedard and Krause, 2007). All of the family members contain several conserved structural features such as binding sites for NADPH, oxygen, and flavin; four heme groups; and six transmembrane domains (Bedard and Krause, 2007; Zekry et al., 2003). Recently, coculture experiments have suggested that microglia NADPH involved in paraquat neurotoxicity (Wu et al., 2005). However, mechanisms whereby paraquat induces dopaminergic neurotoxicity remain unclear. Our data from preliminary studies indicated the ability of paraquat to induce cytotoxicity in SH-SY5Y neuroblastoma cells. In this study, we investigate whether NADPH oxidase may mediate the paraquat cytotoxicity in dopaminergic neurons.

Experimental procedures:

Cell Culture and Chemicals:

Human neuroblastoma cells (SH-SY5Y) were routinely cultured in 75 ml flasks containing DMEM:F12 (Gibco) with 10% FBS and 500 units/ml Penicillin/Streptomycin (Gibco). After confluent, cells were subcultured in 6 or 24-well plates for experiments. The chemicals used in the treatments include: U0126 (promega), GF109203x (Biosource) and gp91ds-tat (Anaspec). The rest of the chemicals used were from Sigma: paraquat, rottlerin and 2',7'- 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Cells were routinely checked for contaminations and other morphological abnormalities prior to use in experiments.

Assessment of cell viability:

The MTT assay was used for determination of cell viability. Before treatment with paraquat and inhibitors, cells were placed in serum-free DMEM. Cells were incubated with paraquat and other agents for different periods up to 48 hours. After various treatments, culture medium was removed and 1 mg/ml MTT dissolved in DMEM was added to the cells and incubated for 2 hours. MTT was converted to blue formazan crystals by the dehydrogenases in active mitochondria in live cells. After incubation, medium was discarded, crystals dissolved in dimethyl sulfoxide:ethanol (1:1, by vol) and absorbance of the solution read at 560 nm (Li et al., 1999).

Membrane Integrity:

Cell viability was determined using LDH assay from Promega. After cells were differentiated days in 24 well plates, the growth medium is replaced with serum-free medium and treated with paraquat. After 48 hours, 50 μL of the medium from the each well of the 24 well plate were removed to a different well of a 96 well plate. The reconstituted substrate mix/assay buffer (50 μL) was added to each of well of 96 well plate and incubated for 30 minutes at room temperature protected from light. Stop solution (50 μL) was added to each well. Absorbance was measured at 490 nm on a Packard Fusion plate reader. (Riss and Moravec, 2004)

Immunoblot assay:

After incubation and treatment, cells were lysed with lysis buffer (150 mM NaCl, 50 mM tris pH 8, 2 mM EDTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS) plus protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) (Ruiz-Leon and Pascul, 2001). The lysate was then centrifuged for 15 minutes at 14,000 rpm. For isolation of membrane fractions, cells were suspended in a membrane separation buffer (0.25M sucrose, 5mM MgCl2, 2mM EGTA, 2mM EDTA, 10mM Tris (pH7.5), 10μg/ml leupeptin, 1mM phenlymethylsulfonyl fluoride, 10μg/ml pepstatin, 10μg/ml aprotinin) and centrifuged at 100,000g for 1 hour. The pellet was resuspended in membrane separation buffer with Triton-x 100 (Shmelzer et al., 2003). Proteins were separated on 10 % dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and transferred to nitrocellulose membranes at 200 mAmps. Membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST) for one hour at

room temperature. Primary antibodies were diluted in 2% milk in TBST. Primary antibodies include the following: polyclonal anti-p67 $_{phox}$ 1:1000 (Upstate), monoclonal anti-β-actin 1: 2000 (Cytoskeleton), and monoclonal anti-PKCδ 1:500 (BD Transduction Laboratories). After incubation for 1 h at room temperature, excess primary antibody was washed off with TBST. Secondary antibodies, either anti-rabbit goat HRP conjugated (Santa Cruz) or anti-mouse goat HRP conjugated (Santa Cruz), were diluted in 2% milk in TBST. After 1h incubation at room temperature, excess secondary antibody was removed by washing with TBST. Bands were visualized with enhanced chemiluminescence (Pierce) and audioradiograph film (SciMart). Density of bands was measured using the Quantity One software.

Immunoprecipitation:

Cells in 100 mm plates were treated with paraquat as described in experiment. Cells were lysed as previously described. The protein concentration was normalized. Cell lysate (200 μL) was combined with 20 μL of immobilized phospho-tyrosine antibody (Cell Signaling) and rocked over night at 4^{o}C. Beads were was 3 times with lysis buffer. Proteins were separated with Western blot as previously described.

rtPCR:

After cells in 100 mm plates were differentiated for 6 days, growth medium was replaced with serum-free medium and paraquat. mRNA was collected using the PARIS mRNA isolation kit from Ambion. The cDNA was amplified by polymerase chain reaction using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen) and

primers for NOX1, NOX2, NOX4, and the loading control GAPDH. The sequence of the primers are as follows: NOX1 Fwd 5' TGG CTA AAT CCC ATC CAG TC 3'; : NOX1 Rvs 5' AGT GGG AGT CAC GAT CAT CC 3'; NOX2 Fwd 5' TGG ATA GTG GGT CCC ATG TT 3'; NOX2 Rvs 5' GCT TAT CAC AGC CAC AAG CA 3'; NOX4 Fwd 5' TGT TGG ATG ACT GGA AAC CA 3'; NOX4 Rvs 5' TGG GTC CAC AAC AGA AAA CA 3'; GAPDH Fwd 5' AGA CAG CCG CAT CTT CTT GT 3'; GAPDH Rvs 5' CCA CAG TCT TCT GAG TGG CA 3'. The products were separated with electrophoresis on 2% agarose gel with ethidium bromide.(Furst et al., 2005)

Statistics:

All graphs were created using GraphPad Prism. Statistics were performed using One-way ANOVA with Newman-Keuls post test to compare all pairs of columns or a paired t test for data sets with only two groups and a 95% confidence interval.

Results:

When treated with retinoic acid for at least 6 days, SH-SY5Y cells become dopaminergic-like cell including expressing dopaminergic neuronal proteins and develop long processes that are axon- and dendritic-like (Fig 1C). After addition of paraquat, the long processes break (Fig 1D). Paraquat doses dependently decreased the cell viability (measured by MTT assay). Paraquat had significantly induced cell death beginning at 500 μM in neurons at 48 h (Fig 1A). Paraquat also induced dose dependent loss in membrane integrity (using LDH assay) and at 500 μM of paraquat the loss of membrane integrity was significant at 48h (Fig 1B). Since both MTT and LDH indicate significant cell death by 500 μM paraquat at 48h, our subsequent studies for cell viability will use 500 μM paraquat at 48 h.

SH-SY5Y cells express the protein kinase C (PKC) isoforms α, δ, ε, ι, λ and ζ (data not shown). Protein kinase C (PKC) delta has been implicated in mitochondrial dysfunction and activation of caspase cascade (Anantharam et al., 2002; DeVries et al., 2002; Leverrier et al., 2002; Shibukawa et al., 2003). The general PKC inhibitor, GF109103x, and the PKC delta selective inhibitor, rottlerin, attenuated the loss mitochondrial function and cell death induced by paraquat (Fig 2). PKCδ, a novel isoform, normally requires diacylglycerol for activation, but it is unlikely paraquat is inducing the production of diacylglycerol. PKC delta can be activated independent of the lipid co-factors by tyrosine phosphorylation (Konishi et al., 1997; Wooten et al., 2001). As shown in Fig 3, 500 μM paraquat induced tyrosine phosphorylation of protein kinase C delta. Paraquat-induced activation of PKCδ causes mitochondria dysfunction and cell

death in SH-SY5Y cells. In microglial cells, PKCδ plays a role in activation of NADPH oxidase. In SH-SY5Y, the role of PKCδ in NADPH oxidase activation has not been determined.

NADPH oxidase is major generator of superoxide in microglial cells (Li et al., 2005). In order to determine the factors involved in cytotoxicity of paraquat, expression of the $p67_{phox}$ cytosolic subunit of NADPH oxidase, was determined in SH-SY5Y cells. SH-SY5Y cells express the $p67_{phox}$ subunit at significantly lower amounts than BV-2 microglial cells (Fig 4) . Unfortunately, the other NADPH oxidase inhibitors, apocynin and DPI, could not be used in these experiments. Because apocynin is toxic to these cells, and DPI interferes with the MTT assay, we used the peptide NADPH oxidase inhibitor, gp91ds-tat. Our lab has successfully used this compound in these cells in an Alzheimer's model, but it did not attenuate paraquat toxicity at 500 μM (Fig 6). At high concentrations, paraquat toxicity may be through the mitochondria dysfunction. Even though the inhibitor for NADPH oxidase did not prevent the toxicity, low concentrations of paraquat (50 μM) did induce the translocation of the $p67_{phox}$ subunit to the membrane (Fig 5). At 50 μM paraquat cell death became apparent after 8 days of treatment (data not shown). It is unclear what the role of NADPH oxidase activation in paraquat toxicity is, but it may be a larger factor in paraquat neurotoxicity at low concentrations.

ERK1/2 can be activated by environmental stresses and oxidative stress (Cuschieri and Maier, 2005; Nishimoto and Nishida, 2006; Roux and Blenis, 2004). Paraquat is known to induce generation of intracellular oxidative stress (Chun et al., 2001). The MEK inhibitor, U0126, attenuated the neurotoxicity of paraquat (Fig 7). ERK1/2 are involved in a wide range of cellular processes including apoptosis and

U0126 may be preventing the apoptotic pathway. We previously suggested the U0126 inhibited NADPH oxidase in microglia cells and superoxide production. The role of ERK1/2 in activation of NADPH oxidase in SH-SY5Y cells is unclear.

Several disease states show upregulation of the NADPH oxidase subunits (Goyal et al., 2004; Vallet et al., 2005; Zhang et al., 2005). Low concentrations of paraquat (50 μM) not only activated NADPH oxidase but also induced the upregulation of expression of the $p67_{phox}$ in SH-SY5Y cells after 24h (Fig 8). In order determine if other subunits of NADPH oxidase were upregulated by paraquat, we examined the mRNA because there are not any commercially available antibodies for the subunits that we have been successful in using. Our data show that SH-SY5Y cell express at least three NOX isoforms, NOX1, NOX2, and NOX4. Paraquat induced upregulation of the mRNA of the NOX2 isoform but not the NOX1 and NOX4 isoforms (Fig 9). Upregulation of the subunits can induce significantly high amounts of superoxide generated (Bedard and Krause, 2007; Bengtsson et al., 2003).

Discussion:

In an epidemiological study in Taiwan, the risk of developing PD was greater in people who had used paraquat than in people who had used herbicides/pesticides other than paraquat (Liou et al., 1997). Paraquat can create intracellular H_2O_2-related reactive oxygen species which may cause apoptotic cell death (Chun et al., 2001). Paraquat dose-dependently induced cell death in SH-SY5Y neuroblastoma cells. The pathway involved in the neurotoxicity induced by paraquat remains elusive.

PKC can be activated after tyrosine phosphorylation. PKC δ can be tyrosine phosphorylated upon treatment of H_2O_2 and is activated by tyrosine phosphorylation independent of lipid cofactors (Konishi et al., 1997; Wooten et al., 2001). Diacylglycerol and other lipid cofactors regulate tyrosine phosphorylation and activation of PKCδ (Benes and Soltoff, 2001; Shibukawa et al., 2003). The phosphorylation sites include two tyrosine phosphorylation sites (Tyr-52 and Tyr-187) in the regulatory region and two (Tyr-512 and Tyr-523) in the kinase domain (Benes and Soltoff, 2001) Tyrosine phosphorylation of PKCδ by receptor and non-receptor tyrosine kinases increases PKCδ enzyme activity in PC-12 cells (Benes and Soltoff, 2001). Src, Fyn, Lyn, Lck, c-Abl, and growth factor receptors increase activity of PKCδ by tyrosine phosphorylation (Benes and Soltoff, 2001; Shibukawa et al., 2003). Tyrosine phosphorylation of the regulatory domain of full-length PKCδ activates caspase-3 and induces apoptosis (DeVries et al., 2002). Our data shows that paraquat can induce tyrosine phosphorylation of PKCδ.

PKC translocates to the membrane, mitochondria, and the nucleus. PKCδ translocates to the plasma membrane where it binds to diacylgycerol (DeVries et al., 2002; Tanaka et al., 2003). PKCδ contains nuclear localization signals and translocate to

the nucleus upon activation (DeVries et al., 2002; Tanaka et al., 2003). PKCδ also translocates to the mitochondria after exposure to cytotoxic stress (Clark et al., 2003). Phorbol ester initiated the translocation of PKCδ to the mitochondria resulting in loss of mitochondrial membrane potential and release of cytochrome c (Benes and Soltoff, 2001; DeVries et al., 2002; Leverrier et al., 2002). Nuclear translocation of PKCδ is necessary for proteolytic activation by caspase 3 (Leverrier et al., 2002; Wang et al., 2003). The caspase cleaved PKCδ is still capable of localizing to the nucleus (DeVries et al., 2002). Mutation of the caspase cleavage site and caspase inhibitors blocked the translocation of PKCδ to the nucleus and inhibited apoptosis (DeVries et al., 2002). Mutations within the nuclear localization signal of PKCδ inhibited nuclear accumulation and apoptosis (DeVries et al., 2002). UV irradiation-induced apoptosis translocated PKCδ to the nucleus and was induced by tyrosine phosphorylation by c-Abl (Wang et al., 2003). Inhibitors of PKC were able to attenuate the cell death induced by paraquat. The PKC inhibitors may have prevented translocation of PKCδ to the mitochondria and nucleus and inhibition of the paraquat induced death.

Paraquat is known to inhibit the complex I in the mitochondria, which results in generation of ROS (Yang and Sun, 1998a). Another component in the generation of ROS recently identified is NADPH oxidase. Animals that are deficient in NADPH oxidase activity showed attenuated toxicity to MPTP, LPS, and rotenone (Casarejos et al., 2006; Qin et al., 2002; Wu et al., 2003). NADPH oxidase is upregulated in PD patients and MPTP treated mice (Wu et al., 2003). The inhibitor of NADPH oxidase was unable to inhibit the toxicity of paraquat. Although, at low concentration paraquat induced translocation of the $p67_{phox}$ subunit to membrane. SH-SY5Y neuroblastoma cells express

significantly less of the $p67_{phox}$ subunit compared to BV-2 microglia cells. This suggests that NADPH oxidase is not major factor the toxicity of paraquat in SH-SY5Y cells.

In the early 20^{th} century, respiratory burst by macrophages had been described but the enzyme involved, NADPH oxidase, was not discovered until the 1980s (Bedard and Krause, 2007). The first catalytic subunit of NADPH oxidase was originally called $gp91_{phox}$ but later changed to NOX2 (Bedard and Krause, 2007; Geiszt, 2006). NOX2 was found in neutrophils and macrophages which is why it also called the phagocytic NADPH oxidase (Bedard and Krause, 2007; Zekry et al., 2003). NOX2 is widely expressed in several cell types such as phagocytes, neurons, thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, testis, cardiomyocytes, skeletal muscle myocytes, hepatocytes, endothelial cells and hematopoietic stem cells (Bedard and Krause, 2007). NOX2 is a heavily glycosylated transmembrane enzyme that transfers electrons across the membrane from NADPH to oxygen through a FAD electron transfer intermediate regulated by $p67_{phox}$ (Bedard and Krause, 2007). The $p22_{phox}$ is necessary for activation of NOX2 and is constitutively associated with it (Bedard and Krause, 2007). NOX2 is found in both intracellular and plasma membranes (Bedard and Krause, 2007). When cells are stimulated, the granules with NOX2 can fuse with the plasma membrane or NOX2 can be activated in the granules (Bedard and Krause, 2007). The role of NOX2 in neurons is not well established because patients and mice lacking NOX2 do not have neurological abnormalities (Zekry et al., 2003). It may play role in neuronal cell death as upregulation of NOX2 correlates with neuronal cell death (Zekry et al., 2003). NOX2 is activated in AD, and NOX2-/- mice attenuated the damage from ischemic stroke suggesting that it may be involved in neurodegenerative diseases (Zekry et al., 2003).

Expression of NOX2 can be induced in various cell types including neurons by such factors as zinc, brain-derived neurotrophic factor, and angiotensin II (Bedard and Krause, 2007; Bengtsson et al., 2003; Zekry et al., 2003). Our data show increased mRNA expression of NOX2 induced by low concentrations of paraquat.

NOX1 was the first homolog of NOX2 identified and has 55% identity to NOX2 (Bedard and Krause, 2007; Bengtsson et al., 2003; Geiszt, 2006). The NOX1 has the most similarity to NOX2 with six transmembrane domains and binding sites and also in the number and length of exons in the two genes (Bedard and Krause, 2007; Bengtsson et al., 2003; Geiszt, 2006). The gene has binding sites for signaling transducers and activators of transcription (STATs), interferon regulatory factor (IRF), AP-1, NF-κb, CREB, CBP/p300 elements and GATA factors (Bedard and Krause, 2007). The enzyme requires the cytosolic subunits, NOXO1 ($p47_{phox}$ homologue) and NOXA1 ($p67_{phox}$ homologue), and the membrane bound subunit, $p22_{phox}$, to be active (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). The highest expression of NOX1 is in the colon but also expressed in smooth muscle, uterus, kidney, stomach, osteoclasts, endothelial cells, placenta, prostate, and retinal pericytes (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). Angiotensin II, platelet-derived growth factor, interferon-γ and prostaglandin F2α upregulates NOX1 expression (Bedard and Krause, 2007; Bengtsson et al., 2003; Geiszt, 2006; Zekry et al., 2003). NOX1 is upregulated by restenosing carotid artery and following castration in the prostate (Bedard and Krause, 2007). Because NOX1 and NOX2 have high similarity in structure and NOX1 is highly expressed in the colon, NOX1 might function in host defense in the digestive tract

(Geiszt, 2006). The mRNA for NOX1 is expressed in SH-SY5Y cells. There was no significant difference in mRNA levels between paraquat treated and controls.

Discovered in 2000, NOX3 shares 58% amino acid identity with NOX2 (Bedard and Krause, 2007; Geiszt, 2006). NOX3 is very structurally similar to NOX1 and NOX2 with six transmembrane domains, binding site, length of extracellular loops, and heme binding histidines (Bedard and Krause, 2007). NOX3 protein or mRNA has been detected in fetal lung, kidney, liver, spleen, skull and brain, but the main expression site is the inner ear (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). Because animals with mutant NOX3 develop balance defects, NOX3 is necessary for vestibular function (Geiszt, 2006). NOX3 requires $p22_{phox}$ for activation (Bedard and Krause, 2007; Geiszt, 2006). It is unclear whether $p47_{phox}$ and $p67_{phox}$ are necessary for activation since depending on the cell type and circumstance, NOX3 can be inactive, weakly active, or active without the cytosolic subunits and $p67_{phox}$ has not be found in the inner ear (Bedard and Krause, 2007; Geiszt, 2006). Unlike the previous two NOX isoforms, NOX3 may be constitutively active and can be further activated by cytosolic proteins (Geiszt, 2006). We have not detected the mRNA of NOX3 in SH-SY5Y (data not shown).

NOX4 has 39% homology to NOX2 especially in transmembrane regions and binding sites for heme, NADPH, FAD (Bedard and Krause, 2007; Geiszt, 2006). Because NOX4 is highly expressed in the kidney, it was originally called renal oxidase or Renox (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). NOX4 is found in neurons, astrocytes, vascular endothelium, smooth muscle, fetal liver, osteoclasts, hematopoietic stem cells, fibroblasts, keratinocytes, melanoma cells, and adipocytes (Bedard and Krause, 2007; Bengtsson et al., 2003; Geiszt, 2006; Zekry et al., 2003). NOX4 requires

$p22_{phox}$ for activation, but does not require the cytosolic subunits or Rac (Bedard and Krause, 2007; Geiszt, 2006). NOX4 has been shown to be upregulated by endoplasmic reticulum stress, shear stress, carotid artery injury, hypoxia and ischemia, angiotensinII, transforming growth factor-β1, tumor necrosis factor-α and streptozotocin-induced diabetic rats (Bedard and Krause, 2007; Geiszt, 2006). Interestingly, when NOX4 is activated, hydrogen peroxide and not superoxide was detected (Bedard and Krause, 2007). This may be because NOX4 localizes to the intracellular organelles where any superoxide produced would get quickly converted to hydrogen peroxide (Bedard and Krause, 2007; Geiszt, 2006). The mRNA for NOX4 is expressed in SH-SY5Y cells. There was no significant difference in mRNA levels of NOX4 between paraquat treated and controls.

NOX5 was discovered in 2001 and has the least homology to NOX2 with only 27% identity (Bedard and Krause, 2007; Geiszt, 2006). There are also other gene products from the NOX5 gene including NOX5α, β, γ, and δ, which contain a long intracellular N-terminus (Bedard and Krause, 2007; Geiszt, 2006). NOX5 does not require the cytosolic subunits to be active (Bedard and Krause, 2007). NOX5 has Ca^{2+}-binding EF-hands region, which is not contained in the previous NOXs and relies on Ca^{2+} for activation (Geiszt, 2006; Zekry et al., 2003). There is a fifth isoform, NOX5ε, which lacks the EF-hand region and is more similar to the other NOXs (Bedard and Krause, 2007). NOX5 is expressed in the testis and developing spermatocytes (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). ROS has been implicated in fertilization of sea urchin eggs (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). NOX5 has also been found in lymphoid tissue, vascular endothelium, smooth muscle, spleen,

ovary, placenta, bone marrow, uterus, stomach and fetal tissue (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). We have not detected the mRNA of NOX5 in SH-SY5Y (data not shown).

There are also two large NADPH oxidase called DUOX1 and DUOX2 (Geiszt, 2006). The DUOXs have 83% homology to each other and the NADPH oxidase portion has 47% homology to NOX2 (Bedard and Krause, 2007; Geiszt, 2006). DUOX enzymes contain a peroxidase-homology domain, which lacks the heme binding region (Bedard and Krause, 2007; Geiszt, 2006). They also have two EF-hands and another transmembrane domain for a total of seven (Bedard and Krause, 2007; Geiszt, 2006; Zekry et al., 2003). Despite co-immunoprecipitating with $p22_{phox}$, DUOXs do not need $p22_{phox}$ or cytosolic subunits to be active, but calcium is necessary for activation (Bedard and Krause, 2007; Geiszt, 2006). Originally named thyroid oxidases, DUOXs are abundantly expressed in the thyroid gland (Geiszt, 2006; Zekry et al., 2003). Patients with mutations in DUOX genes demonstrate hypothyroidism (Geiszt, 2006). DUOX1 is also found in the airway epithelia, pancreas, placenta, prostate and testis (Bedard and Krause, 2007; Geiszt, 2006). DUOX2 is also expressed in the salivary glands, stomach, duodenum, colon, rectum, airway epithelium, and prostate (Bedard and Krause, 2007; Geiszt, 2006). DUOX espression is induced by INF-γ, interleukin-4 and –13, and insulin (Bedard and Krause, 2007). Interestingly, the mature glycosylated DUOX generates H_2O_2 and only partially glycosylated, immature DUOX produces superoxide (Bedard and Krause, 2007).

There were also three cytosolic subunits associated with NOXs: the organizer subunit, $p47_{phox}$ or NOXO1; activator subunit, $p67_{phox}$ or NOXA1; and p40 subunit

(Bedard and Krause, 2007; Bengtsson et al., 2003; Zekry et al., 2003). A small GTPase, Rac1 or Rac2, also is involved in the activation of NADPH oxidase (Bedard and Krause, 2007; Bengtsson et al., 2003). There is also membrane subunit, $p22_{phox}$ (Bengtsson et al., 2003). The first subunit, $p22_{phox}$, was discovered in 1987 and soon after the cytosolic $p47_{phox}$ and $p67_{phox}$ subunits were discovered (Bedard and Krause, 2007). The last subunit, $p40_{phox}$, was found in 1993 (Bedard and Krause, 2007). Once $p47_{phox}$ is phosphorylated, it translocates with $p67_{phox}$ and $p40_{phox}$ where it interacts with the membrane-bound $p22_{phox}$ (Bedard and Krause, 2007). The $p22_{phox}$ stabilizes the cytosolic subunits with the NOX protein, which leads to generation of superoxide (Bedard and Krause, 2007). $p22_{phox}$ is found throughout fetal and adult tissue (Bedard and Krause, 2007). The cytosolic subunits are more selectively expressed. There are two organizer subunits, NOXO1 and $p47_{phox}$, that share 25% homology (Bedard and Krause, 2007). Unlike $p47_{phox}$, NOXO1 is constitutively associated with the membrane (Bedard and Krause, 2007). $p47_{phox}$ is found in myeloid cells, testis, inner ear, neurons, liver, lung, glomerular mesangial cells, endothelial cells and vascular smooth muscle (Bedard and Krause, 2007). NOXO1 is expressed in the colon, testis, small intestine, liver, kidney, pancreas, uterus, and inner ear (Bedard and Krause, 2007). There are also two activator proteins, $p67_{phox}$ and NOXA1, with 28% identity (Bedard and Krause, 2007). $p67_{phox}$ is found in phagocytes, b lymphocytes, glomerular mesangial cells, endothelial cells, neurons, astrocytes, kidney, and liver (Bedard and Krause, 2007). NOXA1 is found in spleen, inner ear, stomach, colon, small intestine, uterus, prostate, lung, thyroid, salivary glands, and vascular smooth muscle (Bedard and Krause, 2007). The $p40_{phox}$ subunit is expressed in phagocytes, b lymphocytes, spermatozoa, hippocampus, and vascular

smooth muscle (Bedard and Krause, 2007). Depending on the subunit, angiotensin II, thrombin, and interferon-γ upregulates them which can translate to significant increase in superoxide production (Bedard and Krause, 2007; Bengtsson et al., 2003).

In conclusion, paraquat induces mitochondrial dysfunction in SH-SY5Y cells. NADPH oxidase is not a major factor in paraquat neurotoxicity at high concentrations, but NADPH oxidase activation is induced by paraquat. Microglial cells produce significantly more NADPH oxidase than SH-SY5Y cells, but the subunits are upregulated in SH-SY5Y cells with paraquat exposure. Because there is increased expressed after 24h of paraquat exposure, NADPH oxidase may play a larger role in paraquat toxicity with longer length of exposure.

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Fig. 1. Cytotoxicity of paraquat in differentiated SH-SY5Y human neuroblastoma cells.

A: Paraquat induced a dose-dependent decrease in cell viability as determined by MTT reduction assay as described in Method (n=3). B: Paraquat induced a dose-dependent increase in loss of membrane integrity determined by LDH assay (n=7). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control. Representative photomicrographs depicting morphological changes in SH-SY5Y cells in serum free medium with or without paraquat (1 mM). C: Control for 24h; and D: paraquat treated for 24 h;. (Magnification: 200x)

Fig. 1

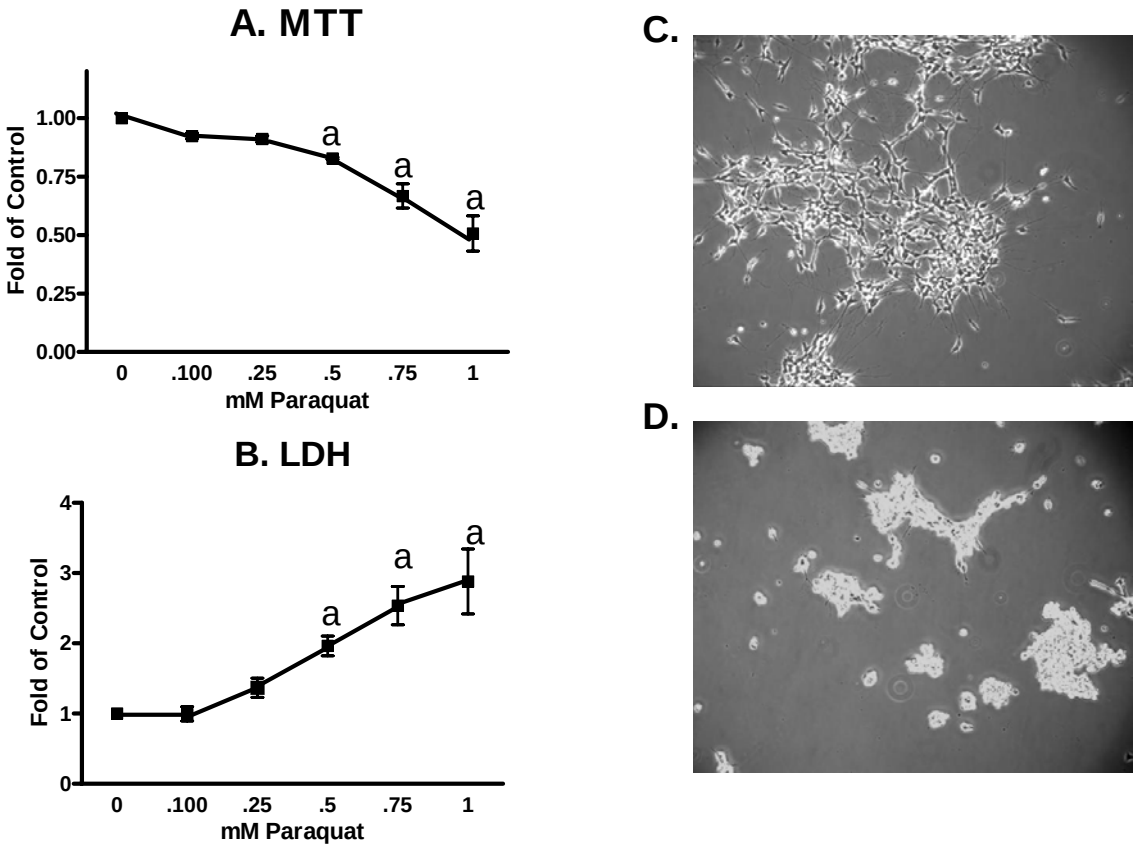

A. MTT
Fold of Control
1.00
0.75
0.50
0.25
0.00
a
a
a
0
.100
.25
.5
.75
1
mM Paraquat
B. LDH
Fold of Control
4
3
2
1
0
a
a
a
0
.100
.25
.5
.75
1
mM Paraquat
C.
D.

Fig. 2. The effects of PKC inhibitors on paraquat toxicity.

Cells were treated with paraquat (500 μM), GF109203x (5 μM), and rottlerin (1 μM) for 48 h as described above. (A) Treatment with paraquat and/or GF109203x for 48 h for cell viability determination (n=3); and (B) treatment with paraquat and/or rottlerin for 48 h for cytotoxicity determination (n=7). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 2

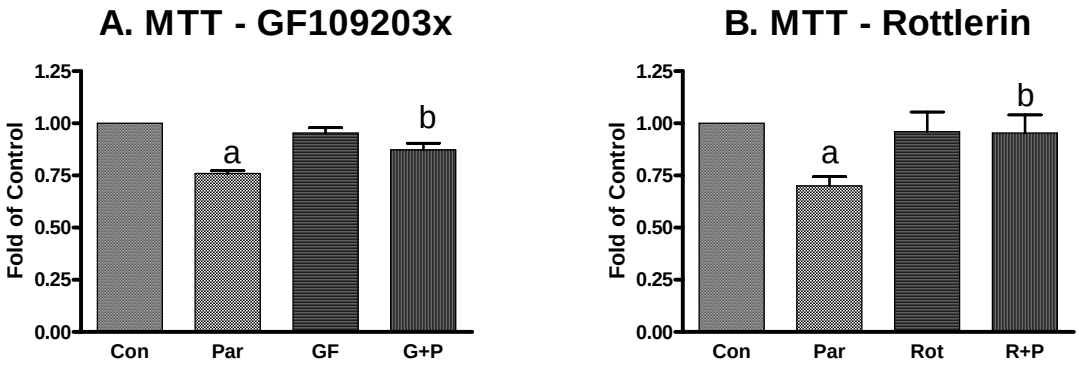

A. MTT - GF109203x
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
GF
G+P
B. MTT - Rottlerin
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
Rot
R+P

Fig. 3. Tyrosine phosphorylation of PKCδ in undifferentiated SH-SY5Y cells.

A. Paraquat (500 μM) time dependently increased the protein kinase C delta phosphotyrosine immunoprecipitation. B. Paraquat (500 μM) time dependently increased translocation of PKCdelta to the membrane. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference ($p < 0.05$): [a] comparing treatment with control.

Fig. 3

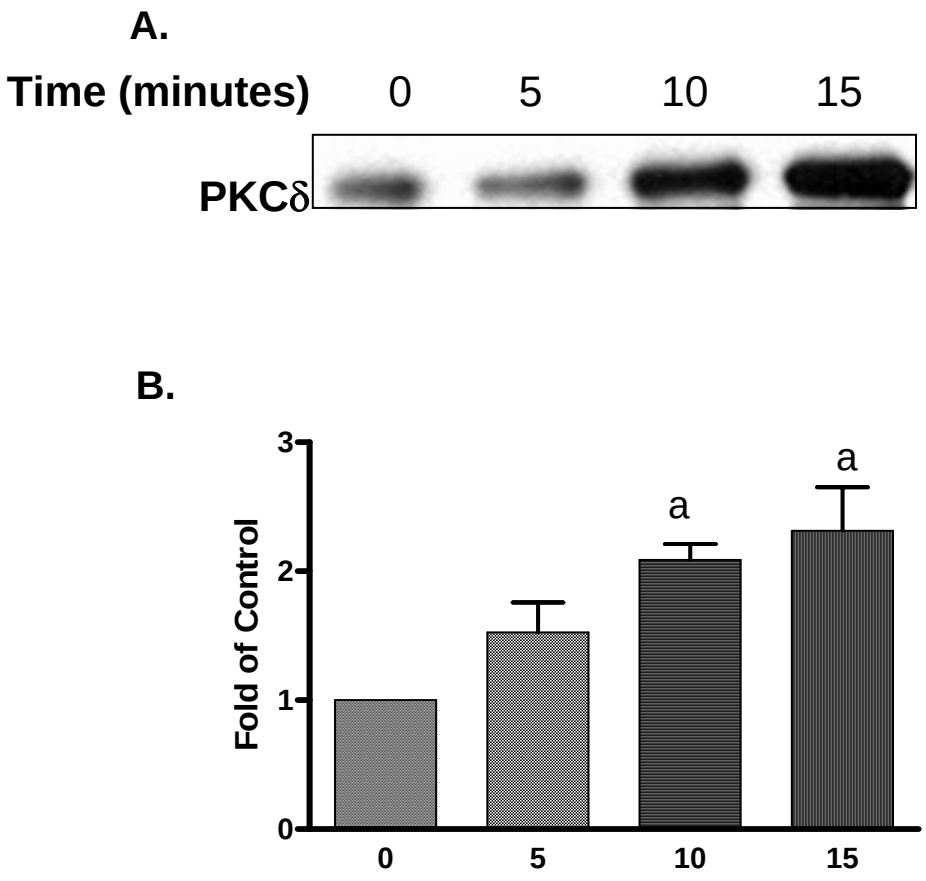


Fig 4. Comparison of NADPH oxidase in SH-SY5Y neuroblastoma cells and BV-2 microglial cells.

Western blot analyses of p67 subunit of NADPH oxidase in BV-2 cells and SH-SY5Y cells (n=3). Beta-actin was used as loading controls.

Fig. 4

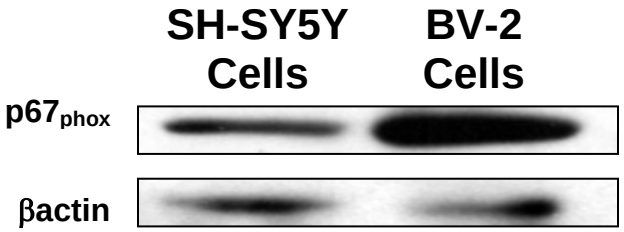


Fig 5. A: Western blot analyses of p67 subunit of NADPH oxidase in cytosol and membrane fractions after paraquat treatment.

Cells were exposed to paraquat (50 μM) for 0, 5 and 10 min after which cells were subjected to fractionation to separate cytosol and membrane fractions as described in text. Beta-actin was used as loading controls. B: Density scan of the blots indicating ratios between $p67_{phox}$ and β-actin in membrane fraction from four independent experiments.

Fig. 5

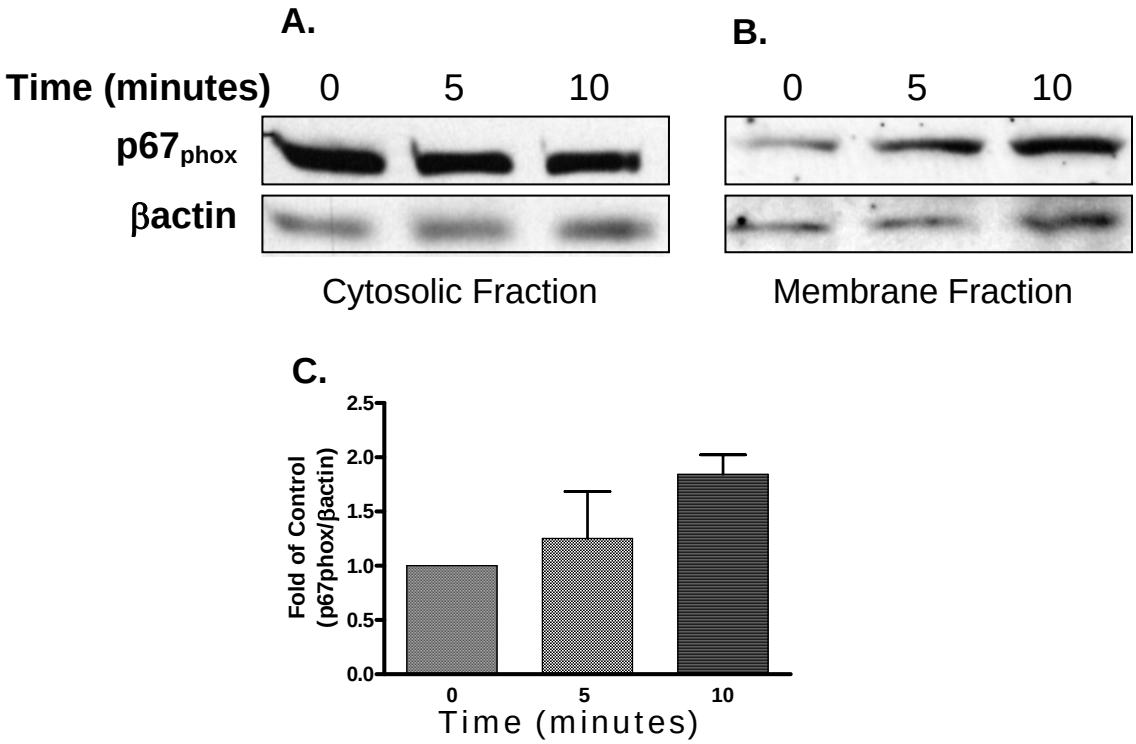

A.
B.
Time (minutes)
0
5
10
0
5
10
p67phox
βactin
Cytosolic Fraction
Membrane Fraction
C.
Fold of Control
(p67phox/βactin)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
Time (minutes)

Fig 6. Inhibitor of NADPH oxidase effect on paraquat neurotoxicity.

Cells were treated with paraquat (500 μM) and/or gp91ds-tat (1 μM), and rottlerin (1 μM) for 48 h for cell viability determination. Results are mean ± SD from 4 independent experiments. One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control.

Fig. 6

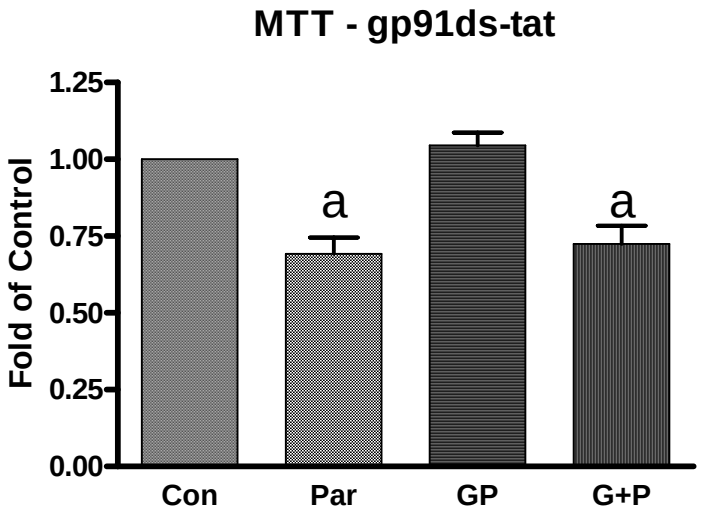

MTT - gp91ds-tat
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
a
Con
Par
GP
G+P

Fig. 7. Phosphorylation of ERK1/2 and effects of MEK inhibitor on paraquat-induced ROS production and cytotoxicity.

(A) For demonstration of ERK activation, cells were exposed to paraquat (50 μM) for 0, 5, 10, 15 and 20 min prior to lysis for Western blot using antibodies for p-ERK and total ERK (n = 3). (B) Cells were exposed to paraquat (500 μM) and/or U0126 (10 μM) for 48 h for assessment of cytotoxicity (n = 3). One-way ANOVA followed by Newman-Keul post-tests indicated significant difference, $p < 0.05$: [a] comparing paraquat with control and [b] comparing paraquat plus inhibitor to paraquat.

Fig. 7

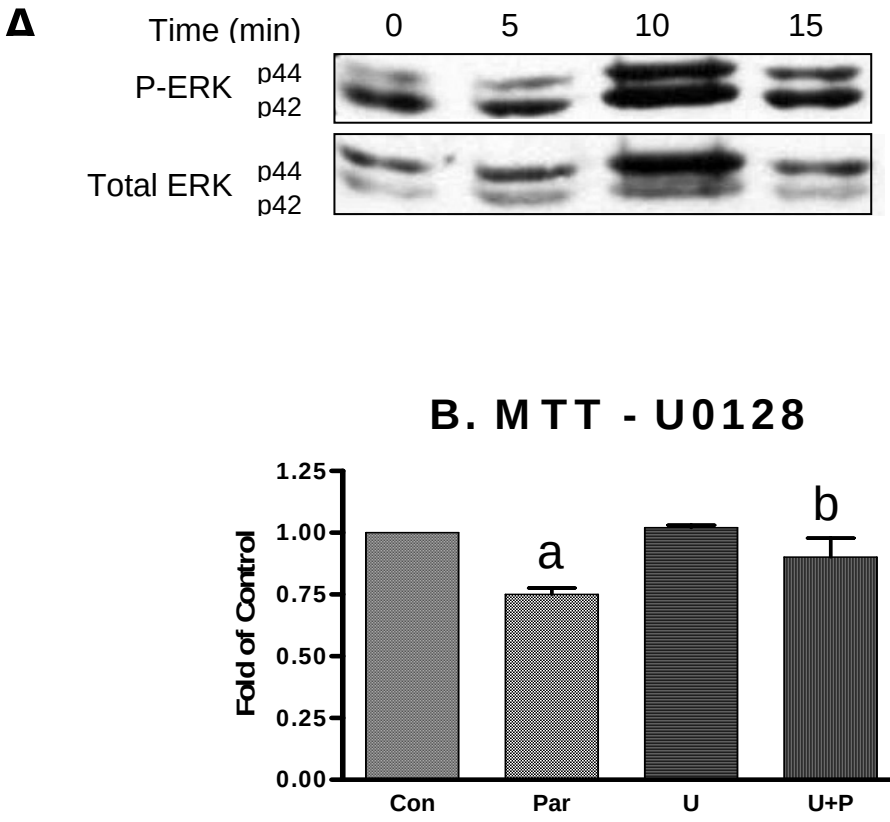
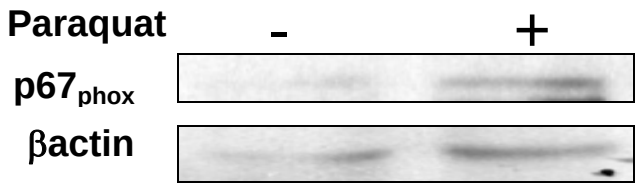

A
Time (min)
0
5
10
15
P-ERK
p44
p42
Total ERK
p44
p42
B. MTT - U0128
Fold of Control
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Con
Par
U
U+P

Fig 8. Paraquat upregulates the $p67_{phox}$ subunit.

A. Cells were exposed to paraquat (50 μM) for 24 hours after which cells were lysed as described. Beta-actin was used as loading controls. B: Density scan of the blots indicating ratios between $p67_{phox}$ and β-actin in membrane fraction from 3 independent experiments. A paired t test indicated significant difference, $p< 0.05$: [a] comparing paraquat with control.

Fig. 8

A.


Paraquat
-
+
p67phox
βactin

B.

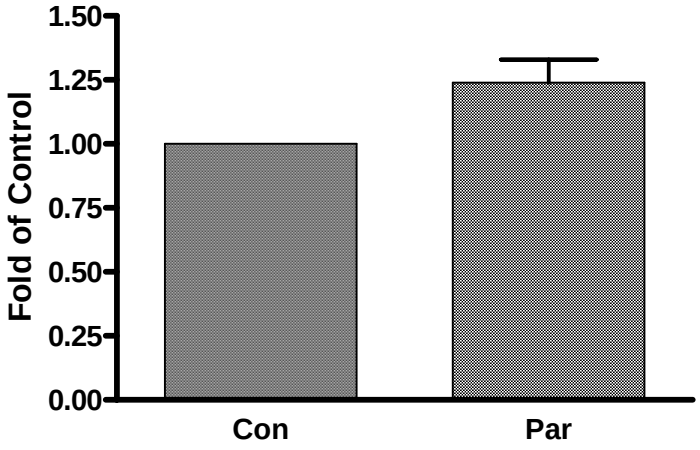
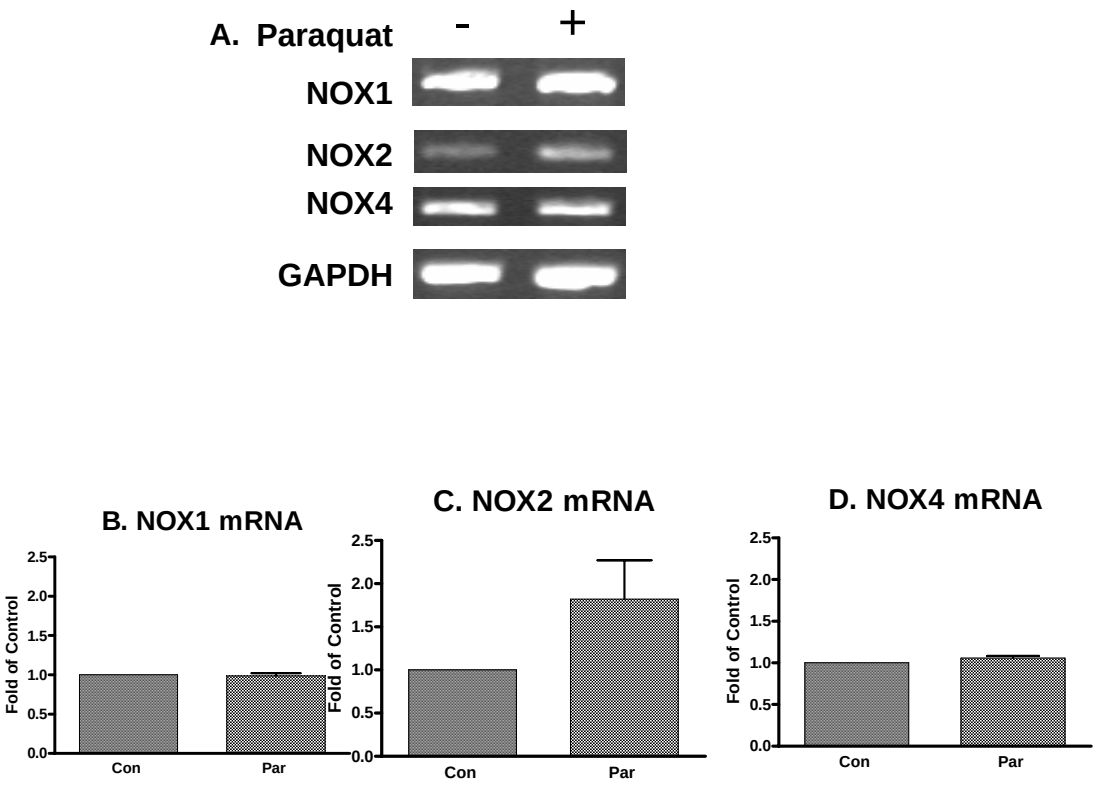

1.50
1.25
1.00
0.75
0.50
0.25
0.00
Fold of Control
Con
Par

Fig. 9. Paraquat upregulates the mRNA of the NOX subunits.

SH-SY5Y cells were treated with paraquat (50 μM) for 24 hours. (A) the mRNA of NOX1 (n=4), (B) the mRNA of NOX2 (n = 3), and (C) the mRNA of NOX4 (n = 4). Results are mean ± SD from number of experiments as indicated. A paired t test indicated significant difference, $p < 0.05$: [a] comparing paraquat with control.

Fig. 9


A. Paraquat
-
+
NOX1
NOX2
NOX4
GAPDH
B. NOX1 mRNA
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
Con
Par
C. NOX2 mRNA
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
Con
Par
D. NOX4 mRNA
Fold of Control
2.5
2.0
1.5
1.0
0.5
0.0
Con
Par

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions:

Parkinson's disease is a debilitating disease that has no cure. The pharmaceutical agents used to treat PD only minimize symptoms and do not stop the progression of the disease. All drugs eventually stop being effective against the symptoms. Understanding the mechanism that leads to the neurodegeneration of the dopaminergic neurons would help develop better strategies that prevent the initiation and progression of it.

Epidemiological studies suggest that paraquat is a risk factor for PD (Liou et al., 1997). Paraquat is an herbicide known to inhibit the mitochondrial respiration chain and thereby, it induces the production of reactive oxygen species (ROS) (Bretaud et al., 2004; Shimizu et al., 2003a; Yang and Tiffany-Castiglioni, 2005). Most of the research has focused on the dopaminergic neurons with limited success. Recently the glia cells have been implicated in the progression of PD.

Microglia cells are the immune cells of the central nervous system and produce ROS when activated (Dringen, 2005; Mander et al., 2006). The substantia nigra, the area affected by PD, has the highest amount of microglia in the brain. Activated microglia are found in PD patients and animal models for PD. Two of the major enzymes for the generation of ROS in microglial cells are NADPH oxidase and nitric oxide synthase (NOS) (Dringen, 2005).

NADPH oxidase is a multisubunit enzyme that generates superoxide (Sumimoto et al., 2004). Mice that lack a functional NADPH oxidase show less neurotoxicity to dopaminergic neurotoxins (Casarejos et al., 2006; Qin, 2004; Wu et al., 2003). Inducible NOS can generate micromolar levels of NO. Inhibition of the NOS attenuated cell death from MPTP and LPS (Gao et al., 2003c).

The focus of this dissertation was to determine the role of microglia in paraquat toxicity. The studies offer original data establishing the activation of microglial NADPH oxidase, NOS, and the signaling pathway activating NADPH oxidase. The dissertation also discusses NADPH oxidase role in the toxicity of paraquat in neuronal cells. The major conclusions are the following:

1. Paraquat exposure significantly increases the generation of ROS and decrease in cell viability in microglia cells. Paraquat induces the activation of NADPH oxidase, which will produce superoxide.

2. Protein kinase C δ and ERK1/2 play a role in the cytotoxicity of paraquat by activating NADPH oxidase and subsequently generation of ROS.

3. Paraquat attenuated the production of nitric oxide by LPS or INFγ, but increase in ROS production and cytotoxicity in microglial cells. This effect of paraquat was not from inhibition of iNOS expression or generation of peroxynitrite. It appears that paraquat may be interacting directly with iNOS.

4. SH-SY5Y cells are less sensitive to paraquat than BV-2 cells. It takes about 10 fold higher concentration to induce cytotoxicity in SH-SY5Y cells than in BV-2 cells.

5. NADPH oxidase is expressed at significantly lower amounts in SH-SY5Y cells. Paraquat also induces activation of NADPH oxidase in SH-SY5Y cells. Inhibition of NADPH oxidase was not protective against paraquat toxicity. NADPH oxidase may not play a major role in paraquat toxicity in neuronal cells.

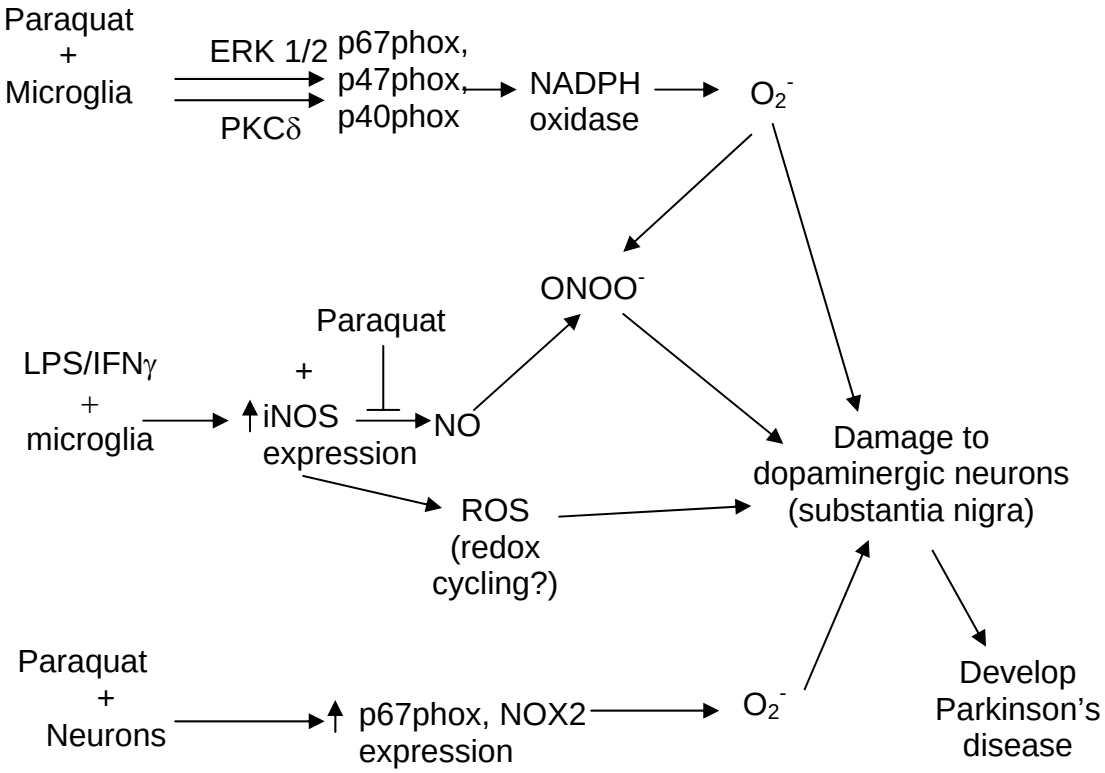
6. Inhibitors for PKC and ERK1/2 did attenuate the neurotoxicity of paraquat. PKC and ERK1/2 have variety of functions in the cells including apoptosis. Inhibitors of PKC and ERK1/2 may be inhibiting the apoptotic pathway in paraquat toxicity.

7. Paraquat induces upregulation of the subunits of NADPH oxidase in SH-SY5Y cells. Increased expression may indicate the role of NADPH oxidase in neuronal cells is significantly increased with exposure to paraquat.

A model illustrating the effect of activated microglial cells induced by paraquat in the substantia nigra of the brain, based the results in this thesis, is proposed in Fig 1.

Fig. 1. Proposed model.

Microglia are in close proximity to neurons. Activation of microglia would affect the surrounding neurons. Paraquat induced the activation of NADPH oxidase in microglial cells. Superoxide generated by microglial NADPH oxidase can diffuse to the neurons and cause damage. PKC and ERK1/2 are involved in the activation of NADPH oxidase. Inflammation increases toxicity of paraquat in microglial cells despite decreasing generation of NO. The increased ROS generation may adversely affect the neighboring neurons. Paraquat may be redox cycling with iNOS.


Paraquat
+
Microglia
ERK 1/2
PKCδ
p67phox,
p47phox,
p40phox
NADPH
oxidase
O2-
ONOO-
Paraquat
+
LPS/IFNγ
+
microglia
iNOS
expression
NO
Damage to
dopaminergic neurons
(substantia nigra)
ROS
(redox
cycling?)
Paraquat
+
Neurons
p67phox, NOX2
expression
O2-
Develop
Parkinson's
disease

Future Directions:

The objective of this dissertation project was to determine the source of ROS generated by environmental toxin like paraquat. The results support the hypothesis that NADPH oxidase is a major generator of ROS induced by paraquat. Likewise inhibiting NADPH oxidase attenuates the cytotoxicity of paraquat. Several questions for future research have been raised by the results presented in this dissertation. The following studies may further determine the role of microglia and NADPH oxidase in Parkinson's disease.

1. Examining the effect on paraquat-induced overactivity of microglia in co-culture with SH-SY5Y cells or in vivo.

The data indicate that paraquat is approximately 10 fold more toxic to microglial cells than to SH-SY5Y cells. Microglial cells are found in the highest levels in the substantia nigra with the dopaminergic neurons (Kim et al., 2000; Lawson et al., 1990). Coculturing the cells together would demonstrate the role play by microglia and the ROS generating enzymes NADPH oxidase and iNOS in paraquat- induced neuronal death. In vivo experiments would also identify the effect of overactivity of the microglial NADPH oxidase on neighboring neurons.

2. Determining the effect of upregulation of the NADPH oxidase subunits.

The data presented show upregulation of two of the subunits of NADPH oxidase in SH-SY5Y cells. Expression of the components of NADPH oxidase can be upregulated

in inflammation and disease states. We propose that increased expression may translate into increased activity. Upregulation of NADPH oxidase proteins significantly increase its activity. Upregulation of NADPH oxidase subunits induced by interferon γ resulted in a fourfold increase in superoxide production (Kuwano et al., 2006). Investigating whether there is a link between upregulation of NAPDH oxidase and increased activity in these cells would better determine the role of NADPH oxidase in neuronal toxicity.

3. Investigating the relationship between environmental toxins and genetic factors.

The data presented demonstrate the toxicity of paraquat in both neuronal and non-neuronal cells. Living in areas paraquat is used is associated with increased risk for PD, but not everyone in the area will develop PD. There are several proteins associated with early onset on PD including Parkin, DJ-1 and α-synuclein (Park et al., 2005; Wang et al., 2005). It has been suggested that development of idiopathic PD most likely depends on both genetic and environmental factors and the genetic factors may also increase the vulnerability of dopaminergic neurons to neurotoxins (Semchuk et al., 1993). Identification of proteins that previously may or may not have been associated with PD but increase neurotoxicity of toxins would help us to develop better strategies for treatment and possibly identifying people at risk.

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