ORGANOCHLORINATED PESTICIDES (OCP) STIMULATED GENERATION OF REACTIVE OXYGEN SPECIES (ROS) IN CULTURED MICROGLIA

By

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To my Mom and Dad, my husband, my baby, and my mentors

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Environmental exposure to pesticides is a possible risk factor to the development of idiopathic Parkinson's disease (PD), a neurodegenerative disorder resulting from the progressive destruction of the nigrostriatal dopaminergic pathway. Multiple lines of evidence indicate the organochlorinated pesticides (OCPs) dieldrin and lindane may be strong candidates that may contribute to PD development. First, the highly persistent and bio-accumulative dieldrin is widely present in the ecosystems and our daily food supply. Both dieldrin and lindane have been found in samples from general population, and it is possible that exposure to combinations of OCPs may increase the risk for PD development compared to exposure to a single OCP. Furthermore, in postmortem PD brains, elevated levels of dieldrin and lindane were detected in substantia nigra.

In recent years, activation of microglia has been proposed to play an important role in dopaminergic neurodegeneration in animal models of PD and in human PD. Microglia can be activated by immunologic stimuli such as bacterial endotoxins and certain environmental toxicants such as the pesticide rotenone. Activated microglia release various neurotoxic factors including free radicals, cytokines and lipid metabolites. These factors impact dopaminergic neurons to induce their degeneration. Of the various neurotoxic factors released from activated

microglia, free radicals, especially reactive oxygen species (ROS) are particularly deleterious to dopaminergic neurons, which are known to be vulnerable to oxidative damage.

In this study, we determined the ability of nanomolar concentrations of dieldrin or combinations of dieldrin and lindane to induce murine microglia to produce ROS. NADPH oxidase is known as a primary protein complex for ROS production in neutrophil. Studies from postmortem human PD brains and animal PD models suggest an important role of NADPH oxidase in dopaminergic neurodegeneration. We determined that NADPH oxidase is a mediator of OCP-induced microglial ROS generation. In addition, GABA receptors are known to be expressed in microglia, we report that GABA receptors may also mediate OCP-induced microglial ROS production in activated microglia.

CHAPTER 1
LITERATURE REVIEW

Parkinson's Disease (PD)

As the second most common neurodegenerative disorder after Alzheimer's disease, there are 1-1.5 million people in the US who are affected by PD (Dowding et al., 2006; Khandhar and Marks, 2007). Loss of the nigral dopaminergic neurons, depletion of neurotransmitter dopamine (DA) in striatum, and the presence of intraneuronal cytoplasmic inclusions (Lewy bodies) are three pathological hallmarks of PD (Takahashi and Wakabayashi, 2001). The typical clinical features of PD in most patients include resting tremor, rigidity in the muscles, slowness in movement and delay in initiating movement, and poor balance (Jankovic, 2008). Although it is clear that two forms of PD cases have been found in human, the underlying cause remains unknown. The early onset form, which accounts for less than 5% of PD cases, is the genetic form with onset at about 35 years of age. More than 95% of cases of PD are sporadic with late onset at 60 years of age. Mutations in proteins such as α-synuclein, parkin, UCH-L1, PINK-1 and DJ-1 lead to the genetic form of PD that is mostly found in familial cases (Bogaerts et al., 2008). Risk factors for sporadic PD include environmental factors, oxidative damage, genetic predisposition and infectious agents contribute to the pathogenesis of PD (Dauer and Przedborski, 2003). With increased research on PD, it appears that the underlying cause of PD may involve more than one single factor in the pathogenesis of PD.

Definition and History

In 1817, a classic medical paper, "An Essay on the Shaking Palsy", was published (Parkinson, 2002). It was in this paper that an English surgeon James Parkinson, firstly summarized and described the clinical characteristics of PD as an age-related movement disorder (Parkinson, 2002). In 1861, Jean-Martin Charcot added more symptoms, including the mask face,

contractions of hands and feet, and rigidity to the clinical description. He gave the name Parkinson's disease to the syndrome to confirm James Parkinson's attribution to medical history (Tan and Shigaki, 2007). The underlying biochemical changes in the brain were identified in the 1950s by Swedish scientist Arvid Carlsson, who discovered dopamine as a neurotransmitter in the mammalian brain and found high level of dopamine in the basal ganglia, which was known as an important area in the brain for motor functions (Carlsson, 1959). Arvid Carlsson won the Nobel Prize in 2000. After discovering of dopamine in the brain, the dopamine precursor L-dopa was developed as a treatment for PD since L-dopa can cross the blood-brain barrier and be converted into DA in the brain (Zappia et al., 2000). L-dopa has been applied as the gold standard therapy on many symptoms of PD.

PD is defined as a progressive movement disorder resulting from a massive loss of dopaminergic neurons in the substantia nigra, progressive loss of dopamine in the striatum, and the presence of intraneuronal proteinaceous inclusions-Lewy bodies (LBs) within the central nervous system (CNS) (Dauer and Przedborski, 2003).

Incidence is defined as the total number of new cases per year. As for PD, there are many reports in different areas of the world and sometimes the rates of incidence vary significantly because of different epidemiological methods, record system, population age level, duration of case assessment and other factors (Twelves et al., 2003). Based on many studies coming from similar record system and reliable methods, the incidence of PD is approximately 16 to 19/100,000 in western countries, except for Italy, which reports the lower number of 8.4/100,000 (von Campenhausen et al., 2005). PD is an age-related neurodegenerative disorder, and incidence increases with age gradually after 55 years of age. The peak incidence is in 70-79 age interval and the average onset age of PD is 60-65 years of age (Twelves et al., 2003). Regarding gender

difference, it has been reported that the onset of sporadic cases in males is slightly earlier than that of females, and there is a slightly higher incidence of PD in male was found compared with that of female (Mehta et al., 2007; Tan et al., 2007).

Etiology and Risk Factors

Although there are many different treatments for PD, there is no cure. In idiopathic cases of PD, the underlying cause remains unclear. As for the basis of PD, it has been discussed and argued for a long time. Several hypotheses have been raised with several possible risk factors including aging, environmental factors, and genetic predisposition with environmental exposure (Warner and Schapira, 2003).

Aging

Aging is a risk factor that has been related to PD since James Parkinson's time. He suggested that PD rarely occurred before 50. What is the role of aging in PD progression clinically and pathologically? Accelerated aging was addressed in the last century to explain a faster rate of clinical progression of PD patients after 50. One of the hallmarks of PD is selective neurodegeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta, and ~80% DA neuronal loss contributes to the major symptoms of PD (Takahashi and Wakabayashi, 2001). The reason why DA neurons in SN are relatively vulnerable compared with other types of neurons in the brain is not clear. However, some specific characteristics of DA neurons may give some clues to the vulnerability. Firstly, DA autoxidation and DA metabolism can generate ROS in SN DA neurons as well as DOPAC and HVA (Chiueh et al., 1993). Alterations in antioxidant defenses, and decreased glutathione (GSH) may cause reduced antioxidant capacity, GSH is known as a scavenger of H_2O_2 (Ng et al., 2007). Moreover, in the SN DA neurons, naturally high levels of iron, neuromelanin and lipids are prone to produce detrimental free radicals (Oakley et al., 2007). Iron is a pro-oxidant that can donate one electron to enhance redox reactions and

catalyze ROS formation from DA or the DA precursor-L-dopa. Furthermore, iron can facilitate lipid peroxidation generating 4-hydroxynonenal to react with proteins forming protein aggregation in the SN DA neurons (Wolozin and Golts, 2002). Neuromelanin is a pigment product that comes from cytosolic non-enzymatic DA oxidation (Prota and d'Ischia, 1993). The SN DA neurons are rich in neuromelanin, and, are capable of storing and releasing iron. A distinct feature of SN DA neurons is their autonomous activation property, which is similar to that of cardiac pacemakers in the peripheral system. In many other types of neurons in CNS, ions go through the membrane of the neuron with sodium channels, while calcium channels are the player in SN DA neurons (Surmeier, 2007). And as a result, these calcium channels are available to open much of the time to enhance calcium influx, and this may accelerate the aging process of SN DA neurons by aggregating mitochondrial calcium as a waste of energy. With advanced aging in SN DA neurons, mitochondria can not work properly and consequently produces free radicals such as reactive oxygen species (ROS) during oxidative stress and calcium accumulation (Levy, 2007). The mitochondria dysfunction is a main possibility of pathogenesis of PD and will be mentioned in detail later. If aging is a direct factor related to the sporadic PD occurrence, is it a single factor without any other factors' involvement? If we suppose it is the case, every individual will finally develop into PD if they live longer enough—longer than 90 years old with around 20% DA neurons in SN. However, except those who have familiar PD because of genetic factors, people chronically developed PD symptoms at different time periods, from 50s to 70s. What is the cause for the different onset age? Mitochondrial DNA (mtDNA) polymorphisms or gene mutations may be involved in these differences. Evidence suggests that different mtDNA polymorphisms are linked with different incidence rates of PD, and the mitochondrial function is influenced by mutations in PD genes such as PINK1, LRRK2. It is hypothesized that, due to the

differences in the aging process, individuals have different risks and onset times to develop PD (Greenamyre and Hastings, 2004; Levy, 2007; Surmeier, 2007). If the aging is associated with a genetic predisposition or exposure to environmental factors, the pathogenesis of the disease will be more complex.

Genetic factors

In contrast to the etiology of idiopathic PD cases, many genes have been identified as a risk factor for genetic PD. These findings not only can be applied to treat those ~5% familial PD patients, they also can shed light to understanding of molecular mechanisms of idiopathic PD. Many PD genes have been discovered and associated with inherited PD (Dauer and Przedborski, 2003; Jain et al., 2005). Some of these genes demonstrated significance in studies of PD pathogenesis, although the two types of PD are different in some clinical or pathological characteristics. The first inherited PD gene identified was an autosomal-dominant gene mutation in alpha-synuclein reported in 1997 in a Greek-Italian family (Polymeropoulos et al., 1997). Since then three substitution mutants-A53T, E46K and A30P of the alpha-synuclein gene *SNCA* have been linked with PD. A duplicate *SNCA* locus will generate three copies of the gene, while triplicate *SNCA* produces four copies and induces earlier incidence of PD in the 20s-30s (Deng et al., 2006). It was found that alpha-synuclein is a major component within the LBs in idiopathic PD (Dickson, 2001). Alpha-synuclein may be directly toxic to neurons or indirectly produced during the course of neurodegeneration. As a protein, it contains an acidic C-terminal, alpha-helical N-terminal and a hydrophobic central component. Although the exact function of alpha-synuclein is not well determined, it is found to be important in lipid uptake, vesicle dynamics, learning process, neuronal plasticity and defense from injury in nerve terminals (Windisch et al., 2007). Moreover, alpha-synuclein deficient mice have been generated which show synaptic deficits. Aggregation and fibrillization of alpha-synuclein in LBs and Lewy neuritis (LNs) may

be an early stage event for both sporadic and familiar PD pathogenesis (Polymeropoulos et al., 1997).

Leucine-rich repeat kinase 2 (*LRRK2*) is another autosomal-dominant gene previously known as PARK8 (Gasser, 2006). Both in the autosomal-dominant form of PD and in the idiopathic PD, mutations in LRRK2 have been involved and the most-common mutant is G2019 substitution (Bonifati, 2007), which is linked to ~5% of inherited PD and 1.5% of sporadic PD cases in the overall population.An even dramatically higher incidence rate in North African Arabs of 37% and 41% respectively is seen. Dardarin is the protein produced by wild-type *LRRK2* and is a member of ROCO family, which belongs to the Ras GTPase superfamily. There are two domains conserved in dardarin, one is a Ras GTPase superfamily member Roc (Ras in complex proteins) domain, another one is a COR (C-terminal of Roc) domain (Mata et al., 2006). Unlike that in alpha-synuclein, *LRRK2* mRNA can be detected in human brains but not in SN. On the other hand, dardarin can be detected in LBs of idiopathic PD. However, neither phenotype nor normal function is clear with LRRK2 (Whaley et al., 2006).

Besides autosomal-dominant genes, there are three important autosomal-recessive genes (*parkin, PINK1 and DJ-1*) related to familial PD. As the most common gene involved in genetic PD currently, *parkin* is closely linked with early onset PD (EOPD) at the prevalence rate of 50% (Djarmati et al., 2004). *Parkin* is also known to be involved in 77% of idiopathic PD individuals with onset ages younger than 20. In contrast to alpha-synuclein, *parkin*-linked PD is not characterized with LBs (Mata et al., 2004). Parkin is a 465 amino acid protein with function as an E2-dependent E3 ubiquitin ligase. Parkin recognizes target proteins to trigger the degradation. Parkin has a variety of substrates including synphilin-1, dopamine transporter (DAT), O-glycosylated alpha-synuclein and monomers of microtubules (α-tubulin and β-tubulin).

Inactivation of parkin reduces UPS-mediated degradation of these substrates. There was no good knockout animal model to represent parkin-linked PD in humans. Interestingly, parkin overexpression was found to have a protective effect on mitochondria swelling and apoptosis in vitro (Hyun et al., 2005).

PTEN-induced kinase-1 (*PINK1*) mutations both in homozygous and compound heterozygous manner were related to both sporadic and familial PD cases (Valente et al., 2004a; Valente et al., 2004b). Ubiquitously brain-expressed PINK1 is a 581 amino acid protein which is present in 10% LBs in the idiopathic PD. PINK1 belongs to the Ca^{2+} / calmodulin family of serine/threonine kinases and binds to mitochondrial membranes with its N-terminal target motif (Gandhi et al., 2006). The reason why this ubiquitous protein is specifically related to PD is poorly understood, while some evidence suggests that PINK1 may work together with parkin together to maintain normal mitochondrial functions (Dodson and Guo, 2007).

Among these five important genes, early onset linked *DJ-1* is least understood (da Costa, 2007). The exact function of DJ-1 remains unclear but it may be related to defense of oxidative stress or mitochondrial damage (Lev et al., 2007; Wang et al., 2006b). In the DJ-1 knockout mice, behavioral studies showed motor impairments. No SN neurodegeneration was found, and DAT reuptake capacity was increased with increased sensitivity to oxidative stress (Kim et al., 2005). Similar to that of PINK1, mutant DJ-1 also interacts with parkin and is stabilized by parkin (Olzmann et al., 2007).

Environmental factors

With increased epidemiological studies of environmental toxins in different PD models, more attention has been paid to environmental factor involvement in PD studies development. The role of environmental factors in sporadic PD was first recognized with the discovery of 1-methl-4-phenyl-1,2,3-tetrahydropyridine (MPTP), which is a byproduct of illicit herion synthesis.

The MPTP PD model is the most studied PD animal model and its neurotoxicity is closely related to the clinical features of PD (Jenner, 2003). Although MPTP is not applied to the environment, epidemiological reports has long speculated that living in a rural environment with farming and drinking well water, or with high risk of pesticide exposure, metals exposure, cigarette smoking habits may play a role in later life time onset of PD (Logroscino, 2005). Many studies have reported a higher prevalence of PD in rural area in the US, Canada, Italy and Finland. Epidemiology reports also have investigated specific occupations, and some occupations including cleaners, cabinet-makers and individuals exposed to magnetic fields have been linked to higher risk of PD (Fall et al., 1999). Interestingly, lower risk to develop PD is associated with individuals in the smoking population compared with that of non-smoking population. The risk varies with duration of smoking, intensity and recentness of nicotine exposure (Thacker et al., 2007). As for pesticide exposure, many human epidemiological and animal studies demonstrate the possibility of association with PD. However, there is no conclusive evidence that specific pesticides either single pesticide exposure or multiple exposure of a combination of agents can cause PD in human populations (Li et al., 2005). With neuropathological studies and toxins animal studies, early life time exposure to environmental toxins results in chronic neurodegeneration in SN before the presence of clinical features (Logroscino, 2005). In the animal models including MPTP administration, N,N'-Dimethyl-4,4'-bipyridinium dichloride (paraquat) and maneb combination, and rotenone exposure, PD neurotoxic features including DA depletion in striatum, tyrosine hydroxylase (TH)-positive DA neuron loss in SN and some clinical characters in behavior studies have been determined with these models in monkey, rat or mice.

On the basis of the reports resulting from experimental neurotoxins such as MPTP and rotenone, as well as case-control studies or case reports of pesticide exposure induced neurotoxicity, several types of pesticides have been implicated in PD. Paraquat is a member of bipyridinium herbicides and paraquat is involved into the PD epidemiological survey is. DA depletion, α-synuclein up-regulation and aggregation and oxidative damage have been detected in animal models after single applications of paraquat (Dinis-Oliveira et al., 2006). Pyrethroids used as household insecticides and insect repellents keeping the sodium channels open in the neuronal membranes and thereby kill insects. Permethrin is a member of pyrethroids class, and has been investigated in the etiology of PD in mouse model. Up-regulation of DAT -mediated increases of DA turnover, reduced mitochondrial function and less motor activity were associated with increased vulnerability of DA neurons in permethrin exposure (Elwan et al., 2006). Organochlorinated pesticides (OCPs) contain at least one covalently bonded chlorine atom in the structure. Some OCPs have significant toxicity to plants, animals, and humans. OCP residues were detected in samples of 20 postmortem PD brain and 14 control cases of matching age at death. A long-lasting residue of DDT (pp-DDE) and dieldrin were found in some cases of PD but only dieldrin was significantly higher in PD brains compared with that of control brains (Fleming et al., 1994). Some OCPs including dieldrin have been banned in many areas of the world after several decades of application. With a highly lipophilic and persistent property and bioaccumulation along the food chain, OCPs are detected in the daily food supply in the US, especially in big size ocean fish. Dieldrin residue is ubiquitous in more than 99% of samples of breast milk from most countries (Fall et al., 1999; Schafer and Kegley, 2002). Due to the neurotoxicity of dieldrin, it is possible that early exposure to dieldrin during the lactation period is associated with late onset of PD in the late adult period due to long-term bio-accumulation in

the fat-rich brain. In these epidemiological studies of the PD- environment relationship, small size sampling and lack of dose reconstruction limit the confirmation of the PD-environment relationship (Dinis-Oliveira et al., 2006; Li et al., 2005). Studies of specific environmental toxins focusing on the molecular mechanisms, pathogenesis and clinical features are necessary to relate specific environmental exposure to PD etiology.

Genetic predisposition and environmental factors

Besides the involvement of a single risk factor, it is possible that sporadic PD is a multi-factorial neurodegenerative disorder based of both genetic predisposition and environmental factors (Warner and Schapira, 2003). According to the pathogenesis of PD, sporadic PD and familial PD may share similar causative pathways. Genes encoding inducible nitric oxide synthase (iNOS), DAT, DA receptors and inherited PD-linked α-synuclein and PINK1 have been investigated as candidates of genetic factors in sporadic PD. For example, PINK1 mutations were widely found in sporadic early onset PD, even in negative family history cases. Transgenic mouse models of α-synuclein suggested that the genetic background increased the DA neuron's vulnerability to the pesticides maneb and paraquat combination (Norris et al., 2007). Inherited PD genes including PINK1, LRRK2 and DJ-1 have been related to mitochondrial dysfunction in SN neurodegeneration as well as pesticides maneb and paraquat combination (Hakansson et al., 2008). Future studies in finding specific genes associated with sporadic PD and genetic predisposition-based environmental toxin models will shed light on the multi-factor hypothesis of PD etiology.

Pathogenesis

The underlying molecular mechanisms responsible for PD neurodegeneration are still unknown, while several potential mechanisms including mitochondrial dysfunction, oxidative stress, protein aggregation and misfolding have been proposed.

Mitochondrial dysfunction

Mitochondria are the most important intracellular organelles for energy generation and have been related with ROS generation (Kim et al., 2006). In physiological state, respiration chain in mitochondria consumes a predominant amount of oxygen (Sherratt, 1991). As the most commonly used PD model, MPTP is selectively detrimental to DA neurons in SN. MPTP is converted to active molecule MPP^+, by glial monoamine oxidase B (MAO-B) and transported by DAT into neurons, to inhibit complex I in the mitochondrial electron transport chain (Kopin, 1987). What is the consequence of complex I inhibition? DA neurons have insufficient energy and generate free radicals such as superoxide, leading to neuronal death in the SN. In post-mortem idiopathic PD brains, complex I deficiency in the SN mitochondria has been identified. Neurotoxin MPTP, rotenone and paraquat induce DA neuron loss through the inhibition of complex I. Somatic mutations of mtDNA have been related to PD by showing that SN neurons from PD brains have a higher deletion of mtDNA. Moreover, a variety of PD genes have been associated with mitochondrial dysfunction in different ways (Mortiboys et al., 2007; Reichmann and Janetzky, 2000; Schapira, 2007). Alpha-synuclein knockout mice are resistant to mitochondrial toxins. Overexpression of α-synuclein in transgenic mice enhances neurotoxicity of MPTP with mitochondrial dysfunction (Lotharius and Brundin, 2002). Parkin, DJ-1 and PINK1 associate with mitochondria in direct or indirect pathways, including protection from apoptosis or reduction of oxidative stress (Harley et al., 1993).

Oxidative stress

Oxidative stress is a pathological condition resulting from insufficient scavenging of reactive oxygen species (ROS) and it is deleterious to cell survival. As previously described, basal level of oxidation in SN DA neurons is relatively higher than other types of cells in the CNS. In particular, oxidation of proteins, lipids peroxidation, and elevated levels of iron and

neuromelanin are common in the SN. As the most important neurotransmitter in the SN, DA is thought to be the source of the free radicals, which are a byproduct of DA metabolism. Dysfunction of mitochondria also facilitates oxidative stress in DA neurons by generation of ROS (Onyango, 2008). Besides the DA neurons themselves, microglia-mediated ROS production also accelerates oxidative damage in neurodegeneration (Park et al., 1999). Proteins, DNA and lipids can be functionally damaged by ROS. Post-mortem analysis has implicated consistent oxidative stress in PD brains (Dexter et al., 1989). Increased oxidative damage also has been found in PD animal models or cell models containing the DJ-1 mutation (Wilson et al., 2003), including rotenone (Sherer et al., 2003) and 6-hydroxydopamine (6-OHDA) exposure (Smith and Cass, 2007).

Protein aggregation

Free radicals from the elevated rate of metabolism and from the enzymatic- and auto-oxidation of DA can damage proteins by causing incomplete formation, mutations, misfolding, denaturing, and oxidation proteins in SN DA neurons. As we know, protein degradation within cells is mediated by autophagy and the ubiquitin–proteasome system (UPS) (Seglen and Bohley, 1992). The autophage mechanism degrades membrane and extracellular components; whle UPS is the main pathway that mediates the degradation of abnormalities in the cytoplasm, nucleus and endoplasmic reticulum of cells. In the hypothesis of protein aggregation mediated PD pathogenesis, proteolytic stress leads to excess expression of unwanted proteins or an impairment of UPS-mediated protein degradation (Lim and Tan, 2007). Accumulated abnormal proteins interact with each other or with normal cellular macromolecules to form aggregation products and inclusion bodies, and induce cell death. In the studies of α-synuclein related PD, failure of UPS to degrade abnormal proteins not only leads to protein accumulation and aggregation, but also alters cellular functions (Fornai et al., 2006), resulting in elevated levels of

truncated, full-length, oligomeric and aggregation of α-synuclein via various post-translation modifications. These modifications including phosphorylation, glycosylation, nitration and/or ubiquitination have been found in SN of sporadic PD (Baba et al., 1998). Moreover, in α-synuclein-linked inherited PD, duplication or triplication of α-synuclein leads to an increase in α-synuclein protein aggregation (Singleton et al., 2003). In addition to α-synuclein, another PD related gene LRRK2 deficiency can cause protein accumulation and LB formation in familial PD.

Symptoms and Diagnosis

With the understanding of hypotheses on PD etiology and pathogenesis, what are the characteristic symptoms of PD patients? Typically clinical symptoms include resting tremor, bradykinesia, rigidity, and postural instability. In severe stages of PD, impaired speech, swallowing difficulty and dementia may occur (Jankovic, 2008). In the absence of a specific biological marker for PD diagnosis, it can be a challenge to obtain a clinically accurate diagnosis of PD in the early stages of the disease progression. And it can be hard to differentiate PD from other similar disorders.

Resting tremor is one of the most recognized symptoms of PD in clinical practice and is related to cholinergic systems overactivity (Smaga, 2003). Almost half of the PD patients present with resting tremor as the first manifestation of PD, however, 15% of patients never develop resting tremors. Resting tremor always starts with a slight shaking in the hand, leg or finger. Sometimes this type of shaking causes a back-and-forth rubbing of the thumb and forefinger. These signs may occur on one or both sides of the body and become more noticeable when the patient is at rest. Resting tremor usually disappears during sleeping (Pare et al., 1990).

Bradykinesia is the most characteristic feature of basal ganglia dysfunction in PD (Berardelli et al., 2001). Bradykinesia, or slow movement, is identified as a slow, shuffling walking with an unsteady gait and stooped posture. Freezing of muscles in leg make it hard to

resume normal movement. It is different from hypokinesia (poverty of movement) and akinesia (absence of movement) (DeJong and Jones, 1971). Bradykinesia is especially distressing because it can make the simplest tasks difficult because of the delay in the initiation of the movement.

Other features of bradykinesia include

- a delay in arresting, decrementing amplitude and speed when repeating a movement;
- drooling due to impaired swallowing;
- loss of facial expression;
- loss of automatic movement.

Bradykinesia is related to striatal DA deficiency in PD and is dependent on the emotional condition of the patient (Kishore et al., 2007).

Rigidity is muscle stiffness and tightness occurring proximally in the shoulders and neck or distally in wrists and ankles. Severe stiffness with pain can limit the range of movements. It also reflects the patient's functional disability. Rigidity is associated with postural deformity, resulting in flexed posture (Prochazka et al., 1997). The biochemical mechanisms of rigidity are associated with DA depletion in striatum.

Postural instability in PD, presents as a loss of balance (Bartolic et al., 2005). Damage in the globus pallidus is thought to be responsible for the postural instability of Parkinsonism. Loss of postural reflexes usually occurs in later stages of PD, resulting in frequent falling.

The assessment of PD is difficult, because symptoms vary in the patient during different times and are influenced by emotional state. Regards to diagnosis, two out of these three specific features, including resting tremor, bradykinesia, and cogwheel rigidity must be present to make a diagnosis of PD (Albin, 2006). However, the diagnosis is complicated by other Parkinsonism diseases. Parkinsonism diseases include primary Parkinson's disease (PD) and secondary parkinsonism, which including drug-induced parkinsonism (DIP), toxin-induced parkinsonism, infectious, metabolic, structural and vascular forms (Galvan and Wichmann, 2008).

Treatments

The severe impairment of PD in patient's quality of life makes it important to treat the disease in an effective way. Current therapies still do not cure the disease, but many potential targets have been studied to treat PD in a more effective way with less side effects.

Drug therapy

Presently it is still difficult to diagnose individuals with pre-symptomatic PD in any degree. Traditionally, drugs used for PD only benefit some restricted symptoms of initiation of PD (Langrall and Joseph, 1971). However, this traditional approach of PD treatment needs to be reevaluated in relation to the issues of whether treatment should be offered at diagnosis. If it is the case, drugs should be applied for initiation of the therapy. PD patients seek medical service because of the symptoms of their disease, and the major early clinical features of PD are associated with motor function deficits caused by the progressive loss of DA neurons in SN. Based on this understanding, relief of symptoms and disease modification are predominant goals of early stage treatment in PD (Pletscher and DaPrada, 1993). In the following paragraphs, several types of drugs in clinical use were discussed, including levodopa, dopamine agonists (DA agonists), anticholinergics, and monoamine oxidase inhibitors (MAO-I).

Levodopa is undoubtedly the most popular and traditional drug available to treat PD. In clinical practice, a marked and sustained response to levodopa strongly supports the diagnosis of PD. Levodopa has been used as first-line therapy for many years, Almost all symptoms of PD are responsive to treatment (Halkias et al., 2007). Rigidity and bradykinesia generally show the most significant improvement with levodopa. Resting tremor varies in response to levodopa, and some other symptoms including postural instability and impaired speech are totally unaffected by levodopa because these symptoms may pathologically result from deficits of several

neurotransmitter systems besides DA system (Battistin et al., 1986; Gerlach et al., 2005; Godwin-Austen, 1980).

In the early stage of PD, levodopa provides a potent and effective response to PD, with a long-duration response that lasts continuously known as “honeymoon period”. However, there are some peripheral side effects including nausea, vomiting and postural hypotension that occur during this period. It is known that levodopa is metabolized by two pathways (Abrams et al., 1971). It is converted into DA by dopa decarboxylase or into 3-O-methyldopa by catechol-O-methyltransferase (COMT). A peripheral decarboxylase inhibitor such as carbidopa can be administrated concurrently to prevent the peripheral production of DA and COMT inhibitors such as tolcapone and entacapone slow down the degradation of levodopa and increase the duration of the drug (Stocchi et al., 2003).

With the progression of PD, many serious and chronic complications appear gradually that impact patients’ quality of life with levodopa treatment (Battistin et al., 1986; Koller et al., 2005; Rabey et al., 2002). These complications consist of “wearing off” and dyskinesia; these affect 75% of patients after several years of levodopa treatment. The “off” condition means a return of all the symptoms even those non-motor symptoms such as depression, anxiety and panic. Dyskinesia is abnormal involuntary movements developed after several years of levodopa treatment. Another problem of levodopa therapy is that some in vitro studies suggested that levodopa may be a neurotoxic molecule to DA neurons and chronic application of levodopa may be harmful to PD patients in terms of disease progression (Gerlach et al., 2005). Presently, there is no conclusion yet, whether levodopa can cause further disease progression. More research on the levodopa molecular mechanisms within the CNS is necessary to make it safer to use in the early stages of PD.

Dopamine agonists were first introduced into clinical practice in the 1970s based on the development of our understanding of DA receptors. There are five subtypes of DA receptors that are divided into two groups. D1-like (D1, D5) and D2-like (D2, D3, and D4) families differ in ability to stimulate or to inhibit adenylate cyclase (AC). Among these DA receptors, D1 and D2 receptors are the two major receptors involved in many physiological or pathological states. Dopamine agonists can directly act on postsynaptic receptors. Dopamine agonists have longer half-lifes, reduce DA turnover, and cause less oxidative metabolism compared with levodopa (De Keyser et al., 1995). Moreover, dopamine agonists are potentially neuroprotective to DA neurons resulting from antioxidative and antiapoptotic features. These advantages of dopamine agonists make them widely used in clinical practice (Brooks, 2000). Currently available dopamine agonists include ergot derivatives bromocriptine and cabergoline, and non-ergot drugs pramipexole and ropinirole. Most of these drugs for PD treatment interact with D2 receptors. As for the safety profile, various dopamine agonists share some common side effects due to peripheral and CNS dopaminergic stimulation.

The first dopamine agonist is bromocriptine, which functions as a D2 agonist with a partial D1 agonist activity at micromolar concentrations and as a D1 antagonist at nanomolar concentrations. Early single drug therapy with bromocriptine generated less motor complications than that of levodopa after several years but also showed less efficacy as well (Godwin-Austen, 1980; Le Witt and Calne, 1981). As for adjunct therapy with chronic levodopa treatment, significant improvement of motor functions occurs in the PD patients. Dopaminergic side effects of bromocriptine include nausea, vomiting, dizziness and orthostatic hypotension as well as hallucinosis and psychosis. Cabergoline is a selective D2 receptor agonist with the unique advantage of a long elimination half-life (~65 hours) and long duration of action. Similar to that

of bromocriptine, cabergoline is also associated with typical dopaminergic side effects. Moreover, it can induce daytime sleepiness.

Pramipexole was clinically approved in 1997 and it is active at D3 receptor with high affinity and at low affinity for D2, D4, 5-HT, and acetylcholine receptors as well. It has shown symptom improvement in MPTP and 6-OHDA animal models. Pramipexole has a very high bioavailability with oral administration (Moller and Oertel, 2000). Ropinirole is a selective non-ergot D2-like receptor agonist with greatest affinity for D3 receptor. Ropinirole monotherapy was effective in treating symptoms in early PD patients in randomized double-blind trials (Giladi et al., 2007; Ponsford, 2001).

Anticholinergics were the earliest type of drugs used in the treatment of PD (Katzenschlager et al., 2003). In PD, DA depletion results in increased sensitivity to cholinergic stimulation. Anticholinergics are used to treat tremors in PD patients, while the underlying mechanisms are still unknown. It is generally suggested that they help to restore the balance between dopaminergic and cholinergic neurotransmitter systems (de Smet et al., 1982). Anticholinergics are recognized as an effective initiation therapy for early onset PD patients with the major problems of resting tremor and impairment of cognitive function, relatively less signs of rigidity, bradykinesia or postural instability. Sedation, memory damage, confusion and psychosis are well-known side effects induced by CNS anticholinergic activity. Besides, CNS side effects, anticholinergics can produce a variety of peripheral effects such as autonomic dysfunction (e.g., dry mouth, orthostatic hypotension) and narrow-angle glaucoma (Katzenschlager et al., 2003). Side effects from application of anticholinergics are common and sometimes severe enough to limit their clinical use for PD treatment.

The monoamines include dopamine, catecholamine, norepinephrine and 5-HT. MAOs are enzymes bound to mitochondrial membrane that catalyze these amines. MAO-B selective inhibitor selegiline is used to treat PD clinically by inhibiting DA catabolism and reuptake (Wessel and Szelenyi, 1992). Not only is the effect on DA metabolism, selegiline itself has neuroprotective effects independent of its MAO inhibitory property (Olanow et al., 1998). Selegiline inhibits apoptosis and reduces oxidative stress from increased antioxidant proteins such as SOD and glutathione. Side effects of selegiline include nausea, dizziness, dry mouth and hallucinations. It is not recommended for afternoon administration because of the risk of insomnia from its amphetamine like metabolites. Selegiline allows a delay in the application of levodopa and permits lower dosage of levodopa in adjunct therapy with reduced "off" state (Lees, 1995).

Surgery

Ablative surgery (thalamotomy, pallidotomy, subthalamotomy) for PD has been the most common approach of surgical treatment for many neurodegenerative disorders. With advances in neurosurgical techniques and a better understanding of circuitry of the basal ganglia, surgery treatments have come back into clinical practice,however deep brain stimulation (DBS) is largely replacing PD surgery today (Balas et al., 2006; Starr et al., 1998). What is the basis of surgery treatment of PD? Scientifically, basal ganglia output is centralized through globus pallidum (Gpi), SN reticulata (SNR) and thalamus, the major output is modulated by direct and indirect pathways (Olanow et al., 1994). The direct pathway involves stimulation from striatum. Activation of the direct pathway inhibits Gpi neurons. On the other hand, indirect pathway stimulates Gpi neurons to counterbalance the effect of direct pathway. In summary, the direct pathway provides positive feedback to motor area while the indirect pathway provides negative feedback. At the beginning of the ablative surgery studies, ablative procedures including motor

cortex ablation, pyramidal tract sectioning of the spinal cord and extirpation of cerebellar cortex had very high risk of morbidity and mortality. The first stereotactic surgery was performed in the 1950s and development in the techniques decreases the risk (Markham and Rand, 1963). Prior to levodopa, thalamotomy was the most common surgery for PD patients. Computed tomography (CT) or magnetic resonance imaging (MRI)-guided technology allowed more precise procedures of thalamotomy in PD patients with the major symptom of resting tremor. The targets of ablation consist of thalamus, globus pallidus internal segment (GPi), external segment (GPe), and subthalamic nucleus (STN). The goal of surgery in PD is to relieve the patients severe problems in functional capacity (Koller et al., 1999). Besides the risk of irreversible ablative procedure, side effects of ablative surgery include visual field deficits, sensory deficits, hemiparesis and death.

DBS therapy was discovered in the 1980s, it is helpful in PD treatment since it was reported that high-frequency stimulation (above 100 Hz) reduces tremors. Compared with ablative surgery, DBS lowers the risk of complications and has several advantages, including no bilateral lesion in the brain, increased efficacy, less side effects and is a reversible procedure (Moro and Lang, 2006). With these advantages, high-frequency stimulation has been applied in ventral intermediate nucleus (VIM) for tremors, in the GPi for levodopa-induced dyskinesias (LIDs), and in the STN for rigidity or bradykinesia. Pallidal DBS is effective for advanced PD with marked improvements of symptoms in motor fluctuations. However, the disadvantages include the high cost of the surgery, repeated surgeries because of device complication or battery replacement every three to five years, and DBS is time-consuming in programming the system, the application of anesthesia, and extension wire implantation (Videnovic and Metman, 2008).

Patients were deemed as DBS candidates with following conditions (Limousin and Martinez-Torres, 2008):

- existence of “on” and “off” states and LIDs
- high enough disability to impair patient’s quality of life
- no contraindications for surgery, no alteration of mental functions, no depression

Physical therapy

Although pharmacological therapies are primarily used to treat PD symptoms, it is not a cure for long-term patients with physical problems. Increased attention has been paid to the potential role of physical therapies including physical exercise, occupational therapy and speech therapy (Boelen, 2007). There is a classic system developed by Hoehn and Yahr with five stages to guide the treatment by physical exercise (Hoehn and Yahr, 1967). Exercise in PD patients is beneficial in the early stage with increased DA release and better absorption of levodopa medication (Ouchi et al., 2001). Trainings on activities of daily living (ADLs) including dressing, eating and cooking are based on the understanding that most frequent falls occur in the kitchen and bathroom. With the education and practice of ADLs, PD patients can improve their quality of life by modifying their activities in a safe way (Beattie and Caird, 1980). Speech therapy is important to maintain self-expression, self-confidence and quality of life in PD patients. Problems consist with dysphagia and dysarthria can be treated from speech therapy with improvements in swallowing and speaking (Schulz, 2002).

Gene therapy

Many studies have reported the potential power of gene therapy in the treatment of PD. Gene therapy with expression of functional enzymes in the cell can be beneficial to symptomatic management of PD. In gene delivery models of PD, several viral vectors are applied including adenoviruses (Ad), herpes simplex viruses (HSV), adeno-associated viruses (AAV) and lentiviruses. To avoid side effects from fluctuation and short steady-state levels of drugs such as levodopa administration, localized gene delivery to SN should be an advance to circumvent the side effects from bioavailability (Eberhardt and Schulz, 2004). Gene transfer also can be used as

a strategy to replace enzymes such as TH and VMAT2 in DA metabolism to enhance functions in the system in early stages of PD. Besides symptomatic management of PD by gene delivery, neuroprotection and reset of neuronal circuitry may be achieved by growth factor transfer or specific gene delivery (Jun et al., 2004).

Potential Therapeutic Targets and Agents

Clinical treatments of PD include pharmacological agents, surgery and physical therapy. However, none of these therapies has proven to change the course of the disease progression. There is a great need for agents that not only treat symptoms but also may slow or halt the neurodegenerative progress. Some recently investigated targets and agents show the promise of neuroprotection. The targets or agents described below have been studied in various PD models in the last several years.

COX-2 is an inducible enzyme that is expressed in response to inflammatory stimuli, cytokines, and mitogens. The promoter region of COX -2 has a NF-κB binding site that is shared with other inflammatory mediators including intercellular adhesion molecule 1 (ICAM-1), IL-2, and IL-8 (Griswold and Adams, 1996). The blockade of microgliosis and inhibition of COX-2 activation was beneficial to DA neurons to protect against the neurodegeneration induced by MPTP. Specific inhibitor of COX-2 has effectively used to treat the MPTP-induced PD model (Reksidler et al., 2007).

IL-18

There are a variety of cytokines such as IL-1, IL-2 and IL-6 that are involved in microglial activation. Recently, IL-18 has been reported to be involved in microglial cells. IL-18 bears similarity with IL-1β in their structure, processing, and proinflammatory properties (Dinarello, 1999). IL-18 has been reported to be involved in brain trauma and hypoxia-ischemia and oxygen-induced neurotoxicity in the immature brain (Calcagni and Elenkov, 2006). In a study

comparing wild-type IL-18 and IL-18 knockout mice in the MPTP model, IL-18 actually decreased both the microglial activation and decreased the loss of DA neurons in SNpc (Sugama et al., 2004).

NADPH oxidase

NADPH oxidase is a superoxide-producing enzyme, which is predominantly expressed in phagocytic cells. It is composed of cytosolic and membrane components. NADPH oxidase can also be expressed in sympathetic ganglial neurons and cortical neurons. In the MPTP model and human PD as well, activated microglial ROS generation is mediated by NADPH oxidase (Gao et al., 2002). NADPH oxidase-deficient mice showed a decrease in number of DA neurons at SNpc compared with that of wild type mice after treatment with MPTP (Wu et al., 2003). All the reports suggested a role for NADPH oxidase in microglial activation in MPTP-induced neurotoxicity.

Tumor necrosis factor-α (TNF-α)

As the key component of cytokines involved in PD, TNF-α is a pro-inflammatory factor that can induce microglial activation (Zujovic et al., 2000). Pathologically, TNF-α can be expressed both in microglial and astroglial cells and plays a role in both neurodegeneration and neuroprotection. It has been reported that IκB-α degradation in microglia was induced by TNF. TNF also has an inhibitory effect on cellular cAMP levels by interaction with adenylyl cyclase. This may be a possible mechanism to maintain NFκB activation and to regulate microglial activation toward a neurotoxic phenotype. The study correlated with the fact that the second messenger cAMP plays a neuroprotective role in diverse in vivo and in vitro models of neurodegeneration (Patrizio, 2004).

There are two receptors for TNF-α, TNF-α receptor 1(TNFR1) and TNF-α receptor 2 (TNFR2). These two receptors have distinct signal pathways (Heller and Kronke, 1994). TNFR1

can activate the apoptosis pathway of cells while TNFR2 works in the opposite way. In a chronic MPTP model of PD, TNFR1 (-/-) and TNFR2 (-/-) mice were investigated (Leng et al., 2005). MPTP-treated mice lacking both TNF receptors (TNFR1 (-/-) and TNFR2 (-/-) can prevent the decrease of dopamine and tyrosine hydroxylase in ST and prevent the loss of dopaminergic nerve terminals and the associated reactive gliosis induced by MPTP. The results suggest that there may be other signal pathways involving both TNFR1 and TNFR2.

Vasoactive intestinal peptide (VIP)

VIP is a neuropeptide with potent anti-inflammatory effects. VIP has 60% structural homology with pituitary adenylate cyclase-activating polypeptide (PACAP) and these two peptides belong to the same family that acts on a family of receptors consisting of VPAC1, VPAC2 and PAC1 (Magistretti et al., 1998). VIP decreased the DA neuronal loss in SNpc and nigrostriatal nerve-fiber loss induced by MPTP administration in mice compared with those of control group (Dejda et al., 2005). VIP can interact with VPAC1 receptor, the mechanism of this effect of VIP involves inhibition of microglial activation and subsequent production of iNOS, TNF-α and IL-1β via the cAMP-dependent pathway in response to the regulation of VPAC1 receptor (Delgado and Ganea, 2003).

(2S,2'R,3'R)-2-(2'3'-dicarboxycyclopropyl) glycine (DCG-IV)

DCG-IV is an agonist for Group II metabotropic glutamate receptors (mGluRs) and has been found to have neuroprotective effect against MPP+-induced neurotoxicity (Matarredona et al., 2001). Another study showed that a single injection with a concentration of 20nmol induced the activation of microglial cells. This activation subsequently led to BNDF mRNA expression in the striatum (DeGiorgio et al., 2002). As a neurotrophic factor, BNDF may mediate the neuroprotective effect of DCG-IV against the neurotoxicity of MPTP (Matarredona et al., 2001). However, the neuroprotective potential of this specific group II metabotropic receptor agonist

requires more studies to demonstrate the pharmacological actions on the CNS in the condition of neurotoxicity.

Genistein

Genistein is the main soybean isoflavones (Wang et al., 2006a). As a structural analog of estrogen, genistein has similar pharmacological functions and has effects on CNS to inhibit the inflammatory response. In the LPS model of PD, genistein protected DA neurons in a dose-dependent manner, genistein blocked microglial activation and pro-inflammatory factors production induced by LPS treatment (Wang et al., 2005). The detailed mechanism responsible for the genistein effect on inhibition of microglial activation remains unknown.

Dextromethorphan (DM)

DM is a widely used drug with the pharmacological action as a nonopiod cough suppressant, while the precise mechanism of this action is unknown (Tortella et al., 1989). On the other hand, DM has neuroprotective effect in some animal models of brain diseases. A recent study on the dopaminergic neurodegeneration showed that DM decreased the loss number of DA neurons induced by LPS (Liu et al., 2003b). The possible mechanism of its neuroprotection involves inhibition of microglial activation and their generation of nitric oxide, TNF-α and superoxide.

Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP)

MnTMPyP is a cell permeable superoxide dismutase (SOD) /catalase analog (Moriscot et al., 2007). Elevated levels of ROS generated in LPS-induced PD model can be reduced by SOD as well as MnTMPyP demonstrating similar function with a neuroprotective effect on DA neurons dose-dependently (Wang et al., 2004). MnTMPyP is more potent than SOD. The exact mechanism remains unclear. Reduction of superoxide free radical and PGE2 in microglial is proposed as a potential mechanism of its action.

Andrographolide

Andrographolide is a diterpenoid and is a primary component of a tranditional herb named Andrographis paniculata. Andrographolide has been widely used for the treatment of inflammatory diseases (Qin et al., 2006). Andrographolide exhibited neuroprotective effect on dopaminergic neurodegeneration induced by LPS by blocking microglial activation and generation of pro-inflammatory factors such as ROS, TNF-α, NO and PGE2 and down-regulation of iNOS and COX-2 expression (Wang et al., 2004).

Naloxone stereoisomers

Recently it has been found that the naloxone stereoisomer [(-)-naloxone] is effective in attenuating the damage of cortical GABAergic and dopaminergic neurons induced by Aβ(1-42) as well as LPS (Liu et al., 2000). The neuroprotective effect of (+)-naloxone involved the blockade of the generation of superoxide free radical in microglia. Compared with (+)-naloxone, (-)-naloxone has more side effects because of its inert antagonism on opioid receptor. In summary, both naloxone stereoisomers can be studied further as potential agents for the therapeutic treatment of PD.

Organochlorinated Pesticides (OCP)

The organochlorinated pesticides (OCP) were among the most widely used pesticides from 1940s to 1990s. They are highly resistant to degradation via biological, chemical and photochemical approaches due to highly hydrophobic and lipophilic properties. Large scale agricultural application of many of the OCPs was curtailed in the 1980s and stopped in the 1990s. However, with the routes of atmospheric deposition and outfalls, the sheer quantities of OCPs used and their extreme persistence in the environment and bioaccumulation along the food chain have resulted in the presence of high levels of OCP residues in soil, water, and daily food supplies throughout the world. It has been found that high concentrations of persistent OCP

contaminated marine mammals, such as cetaceans, worldwide bio-accumulation (Bakan and Ariman, 2004; Bro-Rasmussen, 1996; Tanabe et al., 1994). With the features including long biological half-lives, elevated chemical stability and recycling in the environment, OCP is concentrated in the environment and may be toxic to humans via food chain biomagnification. OCPs such as dieldrin have rarely been studied for their relationship to PD etiology (Dick, 2006). Due to the increasing concerns on OCP contamination globally, many studies began to investigate OCP residues in different areas all over the world (Bakan and Ariman, 2004; Fillmann et al., 2002). Among those OCP members, dieldrin, lindane, and DDT are detected most frequently in higher levels compared with other OCP (Corrigan et al., 1998; Jensen et al., 2001). Although the cause(s) of idiopathic PD remains unclear, environmental exposure to pesticides has been proposed to play an important role in the development of PD (Kamel et al., 2007; Liu et al., 2003a). Animal studies have shown that administration of pesticides paraquat, maneb and rotenone induces degeneration of nigral DA neurons and reproduces behavioral deficits of PD (Betarbet et al., 2000; Thiruchelvam et al., 2000).

Dieldrin and PD

As a powerful insecticide, dieldrin belongs to OCP family, with an epoxide ring structure and six chlorides in the molecule. This makes it highly lipophilic and highly persistent in the environment. It was originally produced as an alternative to DDT in the 1940s, being globally used to control soil pests, tsetse flies and other vectors of tropical disease. However, as an extremely persistent organic pollutant, dieldrin tends to bioaccumulate in the environment via the food chain (Luzardo et al., 2006). How about the toxicity of dieldrin to the environment? It is one of the top 20 toxic compounds to humans and one of the top 12 persistent bioaccumulative and toxic (PBT) compounds. Dieldrin is highly toxic to a variety of animals including humans

through acute or chronic exposure, it was banned in the 1990s in most countries but is still in use in some developing countries (Kanthasamy et al., 2005; Luzardo et al., 2006).

As for the acute toxicity of dieldrin in humans, typical symptoms include nausea, vomiting, headache and coma in severe situations. Dieldrin may be a strong candidate to PD development. Due to the lipophilic property, dieldrin enters the brain tissues by crossing blood-brain barrier, The highly persistent and bio-accumulative dieldrin is widely present as a major contaminating OCP in the ecosystems and our daily food supply (Hickey et al., 2006; Schafer and Kegley, 2002). Dieldrin continues to be detected at elevated levels in serum samples of the general population (Bates et al., 2004; Luzardo et al., 2006). More importantly, dieldrin is found accumulated in the SN of some post-mortem PD brains and not in age-matched control group (Corrigan et al., 2000; Fleming et al., 1994). Dieldrin has also been shown to induce oxidative stress and cause apoptosis of dopaminergic neurons in cell culture models (Kitazawa et al., 2003). Furthermore, in animal models, dieldrin induces oxidative stress in the nigrostriatal dopaminergic pathway and renders the nigrostriatal dopaminergic pathway more sensitive to degeneration induced by known dopaminergic neurotoxins such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) (Hatcher et al., 2007; Richardson et al., 2006). Some evidence of dieldrin-induced neurotoxic mechanisms sheds light to the relationship between dieldrin exposure and PD etiology. In addition to DA production, DA reuptake was also influenced by dieldrin exposure with the finding of elevated DAT binding activity in the striatum of adolescent rats. Furthermore, DA neurons are selectively vulnerable to dieldrin exposure compared to non-DA neurons such as GABAergic neurons both in primary cells and cell lines (Kitazawa et al., 2001). The detailed mechanisms of action responsible for the dieldrin-induced dopaminergic

neurotoxicity and especially its contribution to the development of idiopathic PD in humans remain to be delineated.

Lindane and PD

In the serum samples from normal population, dieldrin is detected with high level as well as gamma-benzene-hexachloride (lindane), which is another highly lipophilic OCP. As for lindane, since 1942, it was distributed globally as a powerful insecticide with a rapid action on the majority of external parasites and can be readily found in the environment (Geyer et al., 1993). Exposure to lindane induces liver dysfunction, altered menstruation and neurotoxicity (Singal and Thami, 2006). The symptoms of lindane-induced neurotoxicity include dizziness, headache, seizure and paraesthesia. Combination of dieldrin and lindane was found in an epidemiological study on a random population (Luzardo et al., 2006), indicating an environmental coexistence of these two OCP members. It is possible that repeated exposure to multiple OCPs may lead to a more potent effect on PD pathological development by chronic intake of combinations of OCPs compared with individual intake of OCP. Furthermore, in postmortem PD brains, elevated dieldrin level is detected in SN compared with that of nonparkinsonian control, and the same has been found for lindane (Corrigan et al., 2000). However, similar to that of dieldrin, the underlying mechanisms of lindane induced neurotoxicity remains unclear.

Microglia

Microglia were first named by Nissl as “Stabchenzellen” (rod cells) in 1899 and were considered to play a role in migration and phagocytosis. Microglia were then regarded by del Rio-Hortega as a resident element of the central nervous system (CNS) (Barron, 1995). During the resting state, the surface antigens of microglia are down-regulated. One obvious feature of microglia is high plasticity with heterogenic distribution and morphology. Microglia are involved

in the development and regeneration of CNS. For development, migratory microglia can respond to injuries and form macrophages, which resemble the globose prototypic microglia in embryonic or postnatal brain. For regeneration, microglia develop into macrophages and form the astrocytic scar to regrow and reconstitute the axonal sprouting.

Activated microglia can release a variety of proinflammatory and cytotoxic factors (Barron, 1995; Mayer, 1998). Activated microglia contributes to the neurodegenerative process through some of the same neurotoxic factors, which damage neurons (Barcia et al., 2004; Mayer, 1998). For PD, these neurotoxic factors included complement proteins, cytokines such as TNFα, IL-1 β, and free radicals. In the 1980s, post mortem analysis detected HLA-DR positive microglia in substantia nigra (SN) of PD brains. Subsequent in vitro and in vivo studies found that more neurotoxic factors like IL-1β, superoxide, TNFα, PGE2 and NO are expressed in activated microglia suggesting the involvement of activated microglia in the neurodegeneration process in PD (McGeer et al., 2003; Zhang et al., 2005).

The cellular protein α-synuclein is a key component of Lewy Body, which is one feature of several neurodegenerative disorders especially in that of PD. Increased α-synuclein can be potentially neurotoxic. Most recently, it has been found that aggregated α-synuclein induced microglial activation and further led to the dopaminergic neurotoxicity through microglial phagocytosis of α-synuclein and subsequent NADPH oxidase activation (Zhang et al., 2005). This pathway was different from that induced by LPS. Although NADPH oxidase activation may be the most important step of this pathway, the detailed mechanisms are still not clear.

Beside LPS, rotenone is another agent that can be used to induce neurodegeneration in animal models of PD. Derived from the roots of *Lonchocarpus* species, rotenone is a natural complex ketone and widely used as a pesticide and fish poison. It specifically interacts with the

NADH-ubiquinone oxidoreductase chain 1 (ND1) and NADH-ubiquinone oxidoreductase Fe-S protein 7 (PSST) subunits of complex I by high affinity to inhibit its activity. With hydrophobic structure, rotenone crosses blood–brain barrier and cellular membranes to enter the brain rapidly and freely and then aggregates in subcellular organelles, such as mitochondria (Uversky, 2004).

In a study by Betarbet and colleagues (Betarbet et al., 2000), Sprague-Dawley and Lewis rats were treated with rotenone in vivo. Rotenone induced typical features of PD that include behavioral, anatomical and neuropathological changes. They also found that rotenone had an effect on complex I, systemically and uniformly throughout the brain to induce a partial defect of complex I. The defects of complex I may be associated with the dopaminergic neurodegeneration in SN and ST.

Administration with nontoxic or minimally toxic concentrations of rotenone induced synergistic neurotoxicity in vitro. The mechanisms involved in this synergistic action of the dopaminergic neurotoxicity included NADPH oxidase activation in microglia and the subsequent release of reactive oxygen species. Cultures from animals with a defect NADPH oxidase did not exhibit synergistic neurotoxicity induced by action of the pesticide rotenone and inflammogen LPS (Gao et al., 2003a). Moreover, similar to that of rotenone and LPS, low doses of MPTP and LPS administration induced selectively synergistic dopaminergic neurotoxicity with the NADPH oxidase activation in microglia and then release of reactive oxygen species. Nitric oxide was also involved because blockade of nitric oxide reduced synergistic neurotoxicity (Gao et al., 2003b).

Increasing reports demonstrated that MPTP induced PD model maybe an age-dependent model. The fact that microglial activation has been found in young mice at 24h and then within 3 days but was not present later than 7 days after MPTP administration showed the opposite result

when compared with older mice who still presented microglial activation 7 days after MPTP administration (Sugama et al., 2003).

With acute and chronic administration of MPTP, although the TH neuronal loss showed significant differences in MPTP treated mice in contrast to control animals, the microglial activation had no significant changes in a young mouse MPTP model. All of these findings pointed out that microglial activation is age-related to the neurotoxicity of MPTP model (Sugama et al., 2003).

Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are a subfamily of free radicals containing one or more unpaired electrons. Both oxygen-centered radicals including superoxide, hydroxyl, peroxyl, alkoxyl, hydroperoxyl and non-radical derivatives of oxygen including hydrogen peroxide, hypochlorous acid, hypobromous acid, ozone, and singlet oxygen can be referred as ROS (Wiseman and Halliwell, 1996). As for oxidative stress, it is defined as the condition of a severe imbalance between ROS/RNS generation and antioxidant defense. When the balance is disrupt with excess ROS, it is possible that oxidative stress may result from different effects such as adaptation to defense oxidative damage, tissue injury via DNA or proteins damage, and cell death by necrosis or apoptosis as well. ROS generation may contribute to the pathogenesis of many diseases by causing (Bandyopadhyay et al., 1999): (1) DNA structural alterations and chromosomal alterations resulting from hydroxyl damage; (2) Participation of signal transduction pathways by hydrogen peroxide with the influence on transcription factors such as NF-κB; (3) Regulation of proteins function and gene expression.

Among these subtypes of ROS, superoxide is ubiquitously formed from normal cellular respiration, mitochondrial electron flux and many other conditions. It is biologically toxic with one unpaired electron to kill invading microorganisms in the immune system (Loprasert et al.,

1996). Under pathological instances, superoxide plays a pro-inflammatory role, mediating autocatalytic destruction of neurotransmitters, lipid peroxidation, up-regulation of surface molecules, and gene expression of pro-inflammatory cytokines (Culotta, 2000). As a reactive free radical, superoxide can be converted into other ROS such as hydroxyl radical, hydrogen peroxide and peroxynitrite to facilitate a variety of oxidative reactions.

NADPH Oxidase

NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) is a multi-subunit enzyme complex, which is able to assemble in the membranes during respiratory burst. NADPH oxidase has the normal function to catalyze the reduction of oxygen and oxidation of NADPH to form ROS. Mutations in NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac are associated with a congenital disorder termed chronic granulomatous disease (CGD) (Segal et al., 2000).

There is increasing evidence on the contribution of NADPH oxidase to PD neurodegeneration both in direct and indirect pathways. Report from PD post-mortem brains demonstrated higher expression of $gp91^{phox}$ subunit of NADPH oxidase in the SN_{pc} (Wu et al., 2003). In 6-OHDA-induced rat PD model, 6-OHDA induced significant increase of ROS production from 6-OHDA autooxidation (Rodriguez-Pallares et al., 2007). Microglial activation was detected in this model, and NADPH oxidase inhibitor apocynin attenuated this activation. Moreover, elevated NADPH oxidase subunit $gp91^{phox}$ and $p47^{phox}$ expression also was reduced by the specific inhibitor Apocynin, suggesting microglial activation may play a role in NADPH oxidase related ROS generation in neuroinflammation process.

In regards to the most representative neurotoxin PD model, MPTP model, both neuroinflammation and oxidative stress are characteristic of MPTP-induced neurodegeneration (Wu et al., 2003). In a MPTP model, NADPH oxidase deficient mice verses wild type mice,

mutant mice demonstrated reduced ROS production, loss of SN DA neurons and protein oxidation compared with that of control mice under MPTP administration. These protective effects of the NADPH oxidase $gp91^{phox}$ knockout model indicate that, the excessive ROS resulted from activated microglia via NADPH oxidase is involved in the neurodegeneration process in the pathogenesis of PD.

In LPS-induced mouse model, NADPH oxidase caused elevated gene expression of cytokine TNFα in the LPS-treated wildtype mice but not in knockout mice (Qin et al., 2004). ROS production was also significantly increased in activated microglia in wildtype mice after LPS administration. The evidence from both PD human samples and animal models suggest an important role of NADPH oxidase in neurodegeneration of PD either by direct interference with SN DA neurons or in indirect impact via activated microglia.

GABA Receptors

As a major inhibitory neurotransmitter, GABA is ubiquitously distributed in the brain. There are two major pharmacologically distinct GABA receptors which have been identified, $GABA_A$ and $GABA_B$ (Chebib and Johnston, 1999). $GABA_A$ receptors are ligand-gated chloride-ion channels. Activation of $GABA_A$ receptors increase the intraneuronal concentration of chloride ion and hyperpolarize the neuron (Whiting, 2003). Ninteen different subunits of $GABA_A$ receptors have been identified, and those include 6 α, 3 β, 3 γ, 3ρ, 1 ε, 1 θ, 1 δ and 1 π. The largest groups of $GABA_A$ receptors are A1a and A2a-GABA receptors, consisting of α_1 or α_2 combined with γ_2- and β-subunit, respectively sensitive to benzodiazepine and nonbenzodiazepine. Not only as heteromers, but $GABA_A$ receptors also can be made up of 3ρ subunits as a homomer. The distribution and abundance of the ε-subunit is not as clear as other subunits. However, it has been found that this subunit is located in the dopaminergic system such

as SNpc (Mohler, 2006). Different subtypes of $GABA_A$ receptors mediate different signaling and have distinct subcellular locations with different pharmacological features.

Metabotropic $GABA_B$ receptors are heterodimeric G-protein coupled receptors, located both pre- and postsynaptically with coupling to G proteins including G_i and G_o to reduce the activity of adenylyl cyclase or enhance the second messenger cyclic AMP production (Emson, 2007). Functional $GABA_B$ receptors are heterodimers consisting of $GABA_BR1$ and $GABA_BR2$ subunits. There are two active isoforms of $GABA_BR1$, 1a and 1b, and no variants of $GABA_BR2$ have been currently identified. Activation of presynaptic $GABA_B$ receptors inhibits neurotransmitter release by suppressing neuronal calcium conductance while postsynaptic $GABA_B$ receptors inhibit adenylyl cyclase with elevated potassium conductance and a consequent hyperpolarization in the neurons.

Compared with $GABA_A$ and $GABA_B$ receptors, the pharmacological functions of $GABA_C$ receptors are barely known. $GABA_C$ receptors also are ligand-gated ion channels and are activated by cis-aminocrotonic acid (CACA) (Zhang et al., 2001). It is well known about GABA receptors' distribution in the main resident neurons in CNS. There is increasing evidence demonstrating that GABA receptors can be expressed in some non-neurons within the CNS, including astrocyte and microglia (Duke et al., 2004). Recent reports on GABA receptor expression started from the evidence that genes encoding GABA receptor-associated protein are expressed in microglia.

CHAPTER 2
GENERAL METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12 (1:1, DMEM/F12) was obtained from Invitrogen (Carlsbad, CA). Dulbecco's Modified Eagle's Medium (DMEM), phenol red free DMEM, phenol red free Hanks balanced salt solution (HBSS), heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline (PBS) and other culture supplements were obtained from Mediatech (Mediatech, Herndon, VA). Lindane was purchased from Aldrich (Aldrich, St. Louis, MO). Dieldrin was purchased from MP biomedicals (MP, biomedicals, Aurora, OH). Poly-d-lysine, cycloheximide, actinomycin D were from Sigma Chemicals (St. Louis, MO). Diphenyleneiodonium (DPI) was obtained from Molecular Probes (Molecular Probes, Eugene, OR). 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was from EMD Biosciences (La Jolla, CA). The Bradford protein assay reagents and bovine serum albumin (BSA) were from Bio-Rad Laboratories (Hercules, CA). Dihydroethidium (DHE) was obtained from AnaSpec (AnaSpec, San Jose, CA). GABA was from Acros (Acros, New Jersey). Picrotoxin and CGP35348 were obtained from Sigma Chemicals (St. Louis, MO). Staurosporine was purchased from Sigma Chemicals (St. Louis, MO). All other reagents for the experiments were purchased from Fisher Scientific (Fisher Scientific, Pittsburgh, PA).

Methods

Microglial Cell Line Culture

The stock of immortalized rat microglial cell line HAPI (highly aggressively proliferating immortalized) were seeded in T75 culture flask from 1st generation of passage. Initially HAPI cells were obtained from mixed rat glial cultures from brains of 3-day-old pups. HAPI cells are round shape in the serum-containing medium, and microglia marker such as OX-42 can stain

HAPI cells. Microglial activation was observed in HAPI cells by LPS induction. (Cheepsunthorn et al., 2001). Based on these understanding, HAPI cells were used as a rat cell line of microglia in this project. HAPI cells were cultured in DMEM containing 5% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin at 37°C and 5% CO2 as previously described (Zhang et al., 2007b). The medium were changed every day, and the HAPI cells will be 90% confluent after 3 days. HAPI cells were observed under the microscope to determine the confluence. With long time observation and detection of HAPI cells, it was found that HAPI cells was in the most stable and healthy state from 4^{th} to 10^{th} passages. The cell density was detected by hemocytometer with trypan blue and dilute into final density with medium for use. In order to obtain the most stable results, for treatment, HAPI cells were seeded at 4×10^4 /well with 100 μL/well in 96-well culture plates from 4^{th} passage and were grown for 48 hours in DMEM containing 5% FBS, 50 U/mL penicillin and 50 μg/ml streptomycin at 37°C and 5% CO2 before treatment.

To study if microglia activation was different in species, the immortalized murine microglial BV2 cell line was used as a mouse cell model. BV2 cells were cultivated in DMEM, with 5% FBS and 50U/mL penicillin/50ug/mL streptomycin (P/S) in T75 culture flask at 37°C, 5% CO_2 incubator in a humidified environment of 5% CO_2 and 95% air for days (Wang et al., 2004b). The cell density was detected by hemocytometer with trypan blue and dilute into final density with medium for use. Similar to that of HAPI cells, BV2 cells were cultured in T75 flask and were used from 4^{th} passage. Comparing with HAPI cells, BV2 cells are smaller in shape, and BV2 cells can use from 4^{th} passage to 15^{th} passage in stable and healthy state. After confluence, BV2 cells were seeded at 4×10^4 /well cell density with 100 μL/well in 96-well plates and grown up for 48 hours in DMEM containing 5% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin at 37°C and 5% CO_2 before treatment.

Primary Microglial and Astroglial Cultures

Compared with the data from cell line models, the data from primary culture are better to represent the real conditions and complex mechanisms in the brain. Primary microglia and astroglia were prepared from the whole brains of newborn 1-day-old Fisher F344 rat pups (Charles River Laboratories, Wilmington, MA) as previously described (Liu et al., 2000). All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Briefly, 6-8 rat pups were obtained from each mom. After growing for 1 day, mechanically triturated whole brains of different Fisher F344 rat pups from different moms were plated at 2×10^7 cells/ 150 cm^2 to poly-d-lysine-coated culture flasks and maintained for 14 days in DMEM/F12 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 100μM nonessential amino acids, 50 U/mL penicillin and 50 μg/mL streptomycin at 37°C and 5% CO_2. Because primary culture can not attach with the plate tightly as immortalized cell lines, poly-d-lysine-coating is necessary to maintain the growing of primary cells. Primary culture grow slower than microglial cell lines such as HAPI and BV2, Medium was changed every 4 days after seeding to maintain a mixed culture of microglia and astroglia. After observing 90% confluent and good shape in the coated culture flasks, primary microglia were separated from the adherent primary astroglia monolayer by a gentle shaking at 150 rpm for 2 hours at room temperature on a shaking platform. After shaking, microglial cells were collected and counted for cell density. Primary microglia at higher density were diluted with 10% FBS DMEM and seeded at 5×10^4/well cell density with 100 μL/well in poly-d-lysine-coated 96-well plates and grown overnight in DMEM with 10% FBS before treatment. After collecting of primary microglia, astroglia were left in the poly-d-lysine-coated culture flasks and obtained by consecutive passaging of the microglia-depleted mixed glial cultures. After the fifth passage, adherent primary astroglia was isolated with primary microglia, and there was almost primary

astroglia left in the flask. The adherent layers of astroglia were detached with trypin-EDTA and reseeded in new flasks. After 2-3 times reseeding procedure, pure astroglia were obtained. Astroglia were seeded at 5×10^4/well cell density with 100 μL/well in poly-d-lysine-coated 96-well plates and grown overnight in DMEM with 10% FBS before treatment. We have consistently obtained nearly pure microglia and astroglia (≥ 98%) as determined by immunostaining for cell type-specific markers as previously described (Liu et al., 2001).

Fluorescence Probes

H_2DCF-DA (DCF-DA) (DCF) (Figure 2-1) was applied to detect intracellular ROS generation in microglia. DCF-DA is a cell-permeant indicator for ROS (Smith et al., 1993). Naturally DCF-DA is nonfluorescent reagent, after intracellular esterases remove the acetate groups of DCF-DA, it will be converted into the highly green fluorescent 2',7'-dichlorofluorescein (DCF^+) and detect oxidation within the cell with excitation at 485 nm and emission at 528 nm (Figure 2-2). Here DCF-DA was chosed as a non-selective reagent to detect ROS generation with the ability that many subtypes of ROS can be detected by using DCF-DA. These ROS subtypes include hydrogen peroxide, peroxyl radical (alkylperoxyl and hydroperoxyl), and peroxynitrite anion. In the study of ROS generation in microglia, first question raised was that whether increased ROS generation is induced by OCP. To detect the major amount of intracellular ROS generation, DCF-DA was a good candidate to detect different subtypes of ROS in one experiment. DCF-DA is a light-sensitive powder and can be dissolved in ethanol. With pilot studies on series concentrations (5, 10, and 20 μM) of DCF-DA, DCF-DA at 10 μM was an optimal concentration to detect fluorescent signal with sensitivity at 50 by using a Synergy HT multi-well plate reader.

Dihydroethidium (DHE) (Figure 2-3) was applied to detect oxidative activities in living cells, and selectively detect superoxide production (AlMehdi et al., 1997). DHE is a blue

fluorescent and turn into ethidium after detecting oxidation in the cell. Ethidium intercalates with DNA and become a red fluorescent with excitation at 520 nm and emission at 590 nm (Figure 2-4). With the selectivity on superoxide anion detection, DHE became a sensitive probe for specific ROS subtype superoxide detection in the second aim of the project. With the results from first aim, detection on specific ROS is suitable to determine the role of certain ROS subtype in the ROS generation and is helpful to understand the underlying mechanisms. DHE need to protect against light and will be more light sensitive in solution. With pilot studies on series concentrations (10, 20 and 50 μM) of DHE, DHE at 20 μM was an optimal concentration to detect fluorescent signal with sensitivity at 50 by using a Synergy HT multi-well plate reader.

DCF Assay for ROS Detection

The levels of several subtypes of ROS in cells following treatment were determined using the fluorescent probe DCF-DA as previously described (Gao et al., 2003a). For each DCF assay, a fresh stock solution of DCF-DA (10mM) were prepared in ethanol and kept out of light until ready to use. For treatment, after cells growing confluence in the culture falsk in DMEM with 10% FBS, HAPI, BV2, primary microglial cells, or primary astroglial cells were switched to and maintained for 1 hour at 37°C and 5% CO_2 in serum-free and phenol red-free DMEM (treatment medium). Afterwards, cells were treated (6 wells/condition) at 37°C and 5% CO_2 for desired time intervals with different treatment groups according to the experimental design. At the end of treatment, cells were washed three times with HBSS (100 μL/well), incubated for 1 h with 10 μM light-sensitive DCF-DA diluted in HBSS (100 μL/well), and then washed twice with HBSS (100 μL/well) and switched to and maintained in HBSS (100 μL/well) for reading. Fluorescent intensities were measured at 0, 60 and 120 min after the last HBSS wash at 485 nm (excitation)/528 nm (emission) using a Synergy HT multi-well plate reader (BioTek Instruments, Winooski, VT).

DHE Assay for Superoxide Detection

DHE is a sensitive fluorescent probe frequently used for intracellular superoxide detection (Ganesan et al., 2006). For each DHE assay, a fresh stock solution of DHE (20mM) were prepared in ethanol and kept out of light until ready to use. For treatment, after cells growing confluence in the culture falsk in DMEM with 10% FBS, BV2 microglial cells were switched to and maintained for 1 hour at 37°C and 5% CO_2 in serum-free and phenol red-free DMEM (treatment medium). Afterwards, cells were treated (6 wells/condition) at 37°C and 5% CO_2 for desired time intervals with different treatment groups according to the experimental design. At the end of treatment, cells were washed three times with HBSS (100 μL/well), and incubated for 30 min with 20 μM light-sensitive DCF-DA diluted in HBSS (100 μL/well). After incubation, BV2 cells were washed twice with HBSS (100 μL/well) and switched to and maintained in HBSS (100 μL/well) for reading. BV2 cells were examined in a Synergy HT multi-plate reader (BioTek, Instruments, Winooski, VT) at 0, 10 and 30 min time points. With pilot study, superoxide production increased faster between 0 to 10min interval compared with that between 10 to 30 min, therefore the measurement of fluorescent intensities in the second aim with DHE assay was showed with data for 10 min reading at 520 nm (excitation)/590 nm (emission) (each experiments detected at 0, 10 and 30min reading and only data of 10min reading showed in the figures).

Protein Assay

At the end of treatment, cells were washed twice with PBS, solubilized in 2 N NaOH (100 μL/well) and then kept overnight at − 20°C. For protein analysis, 10 μL of lysate from each well was mixed with an equal volume of 2 N HCl and protein content was determined with the Bio-Rad Bradford protein assay reagents using BSA as standard following the manufacturer's suggested protocol.

Statistics

For each experiment, 6 replicate wells of cells in a 96-well plate were used for treatment for each condition. Relative fluorescence units (RFU) of cells in each well, indicator the amount of ROS produced, were read 2 hours after DCF loading or 40 minutes after DHE loading. RFU values of the replicate wells of each condition (6 for each condition) were averaged. The mean FRU of vehicle control was then subtracted from that of drug-treated cells in the same experiment. The mean RFU values (with control subtracted) from 4-6 separate experiments (ie, n = 4-6 experiments) were then used for analysis of statistical significance. The significant of differences was assessed using ANOVA and Fisher's PLD post hoc with the JMP IN program (SAS Institute, Cary, NC), p value of < 0.05 was considered statistically significant.

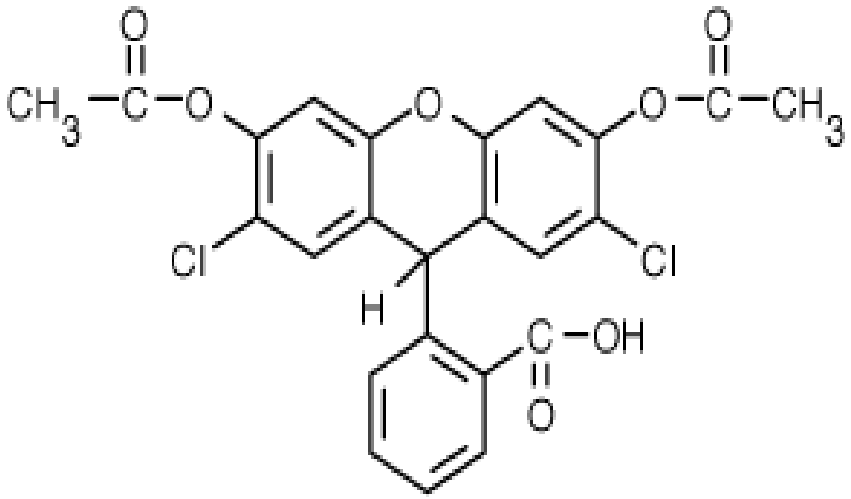


Figure 2-1. Chemical structure of H_2DCF-DA. DCF-DA is an intracellular ROS indicator.

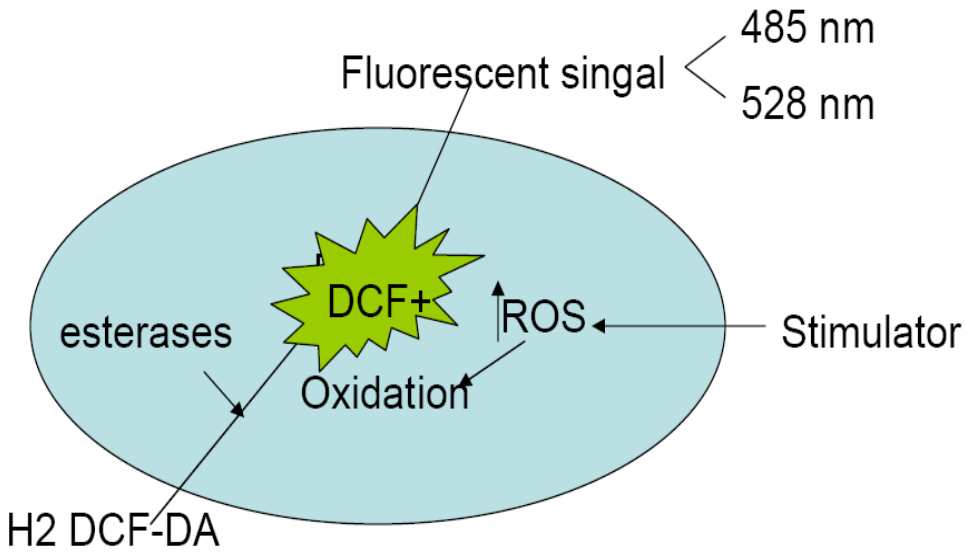


Figure 2-2. The mechanism of action of DCF-DA in the cell. After stimulation, microglia was activated and generated increased ROS. DCF-DA entried into the cell and became DCF+, consequently DCF+ detected oxidation in the cell and release fluorescent singal.

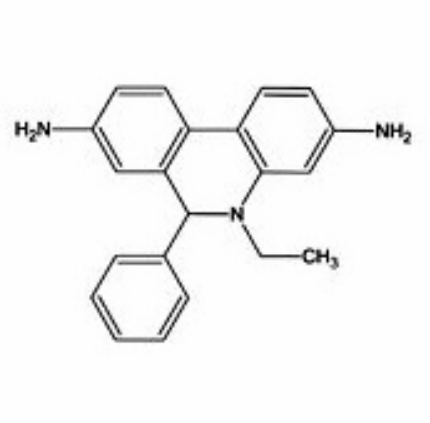


Figure 2-3. Chemical structure of DHE. DHE is a superoxide indicator.

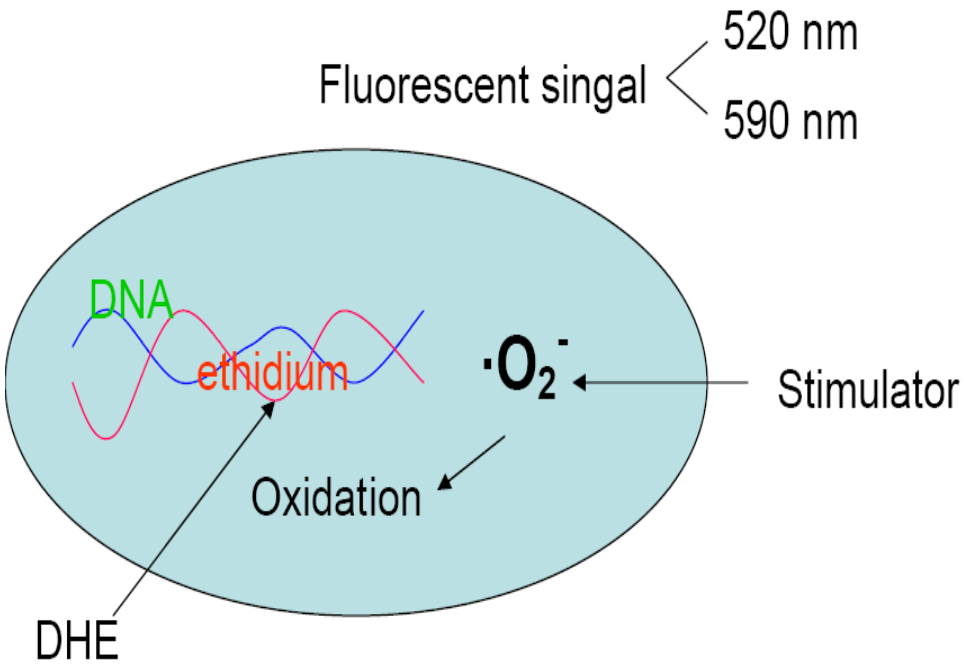


Figure 2-4. The mechanism of action of DHE in the cell. After stimulation, microglia was activated and generated increased superoxide anion. DCF-DA entried into the viable cell and became ethidium, consequently ethidium binds with DNA in the cell and release fluorescent signal.

CHAPTER 3
AIM ONE: EFFECT OF ORGANOCHLORINATED PESTICIDE DIELDRIN ON MICROGLIAL REACTIVE OXYGEN SPECIES GENERATION

Introduction

As the second most common neurodegenerative disorder, PD is characterized by massive loss of SN DA neurons and depletion of striatal dopamine. Although the etiology of PD is unclear, environmental exposure to pesticides is investigated as a risk factor related to the sporadic cases of PD.

Multiple lines of evidence indicate that, among the various suspected pesticides, the organochlorinated pesticide (OCP) dieldrin may be a strong candidate that may contribute to PD development (Kamel et al., 2007; Liu and Hong, 2003). First, the highly persistent and bio-accumulative dieldrin is widely present as a major contaminating OCP in the ecosystems and our daily food supply (Hickey et al., 2006; Schafer and Kegley, 2002). Second, dieldrin continues to be detected at elevated levels in serum samples from the general population (Bates et al., 2004; Luzardo et al., 2006). More importantly, dieldrin has been detected in the substantia nigra of some postmortem PD brains (Corrigan et al., 2000; Fleming et al., 1994). Third, dieldrin has been shown to induce oxidative stress and cause apoptosis of dopaminergic neurons in cell culture models (Kitazawa et al., 2003). Furthermore, in animal models, dieldrin induces oxidative stress in the nigrostriatal dopaminergic pathway and renders the nigrostriatal dopaminergic pathway more sensitive to degeneration induced by known dopaminergic neurotoxins such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) (Hatcher et al., 2007; Richardson et al., 2006). However, the detailed mechanisms of action responsible for the dieldrin-induced dopaminergic neurotoxicity and especially its contribution to the development of idiopathic PD in humans remain to be completely delineated.

In recent years, an increasing number of studies strongly suggest that the activation of the resident brain immune cells, microglia, plays an important role in dopaminergic neurodegeneration in animal models of PD and in human PD (Liu, 2006; McGeer and McGeer, 2004). Microglia can be directly activated by immunologic stimuli such as bacterial endotoxins and certain environmental toxicants such as the pesticide rotenone. Conversely, microglia can be indirectly activated in response to neuronal injuries induced by certain neurotoxins such as MPTP (Liu et al., 2003a; Liu and Hong, 2003). Activated microglia produce a variety of pro-inflammatory and neurotoxic factors that include cytokines, free radicals and lipid metabolites that impact neurons to induce neurodegeneration. Of the various neurotoxic factors released from activated microglia, free radicals, especially reactive oxygen species (ROS) are particularly deleterious to dopaminergic neurons, which are known to be vulnerable to oxidative damage (Sherer and Greenamyre, 2005). Therefore, inhibition of microglial ROS generation by pharmacologic inhibitors or gene deletion of the microglial ROS generating enzyme NADPH oxidase protects dopamine neurons from toxin-induced neurodegeneration (Liu et al., 2000; Qin et al., 2004; Wu et al., 2003).

This study was designed to determine the ability of nanomolar concentrations of dieldrin to induce murine microglia to produce ROS.

Methods

Experimental Design Rationale

Based on the understanding of microglia and OCP member dieldrin, the first hypothesis in this aim was proposed. The hypothesis is that dieldrin would be able to induce increased ROS generation in microglia. Rat microglial cell line HAPI was selected to detect ROS generation because preliminary data from treatment of higher concentrations of dieldrin (10 to 100 μM level) in 2 hours showed increased ROS in HAPI cells (data not shown in the aim). Here the hypothesis

focused on low concentrations of dieldrin (from concentrations of subnanomolar to low micromolar levels), and the treatment time was set with long-term induction (6 hours to 24 hours) in serum-free medium. If the treatment was longer than 24 hours, for instance, 48 hours treatment, the microglial cells wound die because of lack of serum.

Cell numbers for each treatment group were determined by protein assay to determine if 24 hours treatment of dieldrin at micromolar levels is toxic to microglial cells.

After determining the effect of dieldrin on immortalized rat microglail HAPI cells, rat primary microglia were prepared to further investigate the role of dieldrin in primary culture. Astroglia are another glial cell type in CNS with function as trophic support for neurotransmitter reuptake and trophic factors expression. If microglia are a major player of inflammation-mediated neurodegeneration, primary astroglia should be not involved in increased ROS generation. Therefore, primary microglia and primary astroglia were tested for ROS generation side by side to compare their different properties.

A question was raised from the result of ROS generation in HAPI and rat primary microglia: was the dieldrin-induced ROS generation unique to rat microglial cells? In studies on neurodegeneration model, KO mouce model was developed (Forntaine et al., 2002), while no KO rat model in neurodegeneration studies currently. Moreover, in the most popular PD model-MPTP model, there was no effect found in rat, but in mouse, monkey and human, PD features were induced. Therefore it is suitable to detect ROS production in a mouse microglial cell line. Here BV2 cell line was applied as an immortalized mouse microglia.

Treatment

For each treatment, a stock solution (10 mM) of dieldrin was freshly prepared in absolute ethanol. Microglial cells (HAPI, BV2, or primary microglia) or primary astroglial cells were prepared and seeded into 96-well plate on day 0 in the DMEM with 5% FBS (for immortalized

microglial cells) or 10% FBS (for primary cells) (Figure 3-1). Primary cells required more serum in the medium to maintain stable and healthy conditions. As for immortalized microglial cells, HAPI or BV2 were seeded at 4×10^4 /well cell density with 100 μL/well medium at 37°C and 5% CO_2, 48 hours later, the cells will reach 90% confluence in wells. Compared with the immortalized cells, the primary cells grew slower in the medium, since microglia or astroglia were seeded at 5×10^4 /well cell density with 100 μL/well medium at 37°C and 5% CO_2, 48 hours later, the primary cells will also reach 90% confluence in poly-d-lysine-coated wells. After 48 hours growing in the serum medium for confluence, in order to decrease the influence from other factors in the serum such as growth factors, cells were switched to and maintained for 1 hour at 37°C and 5% CO_2 in serum-free and phenol red-free DMEM (treatment medium). Afterwards, cells adapted to the treatment medium were treated (6 wells/condition) at 37°C and 5% CO_2 for desired timed intervals (6, 12, or 24 hours) with vehicle control (0.01% ethanol in medium) or desired concentrations of dieldrin diluted in the treatment medium. Without any treatment of dieldrin or other stimulator, generally microglia are capable of release small amounts ROS as a normal function. There were no differences in ROS generation between vehicle control and medium control (only treatment medium). Vehicle treatment was used as control in data analyses because dieldrin powder was diluted into ethanol as the fresh stock and then dilute into final concentration in medium for each treatment, it is better to delete the influence from vehicle by using vehicle control during data normalization. After treatment, DCF-DA at 10 μM was loaded for 1 hour and washed off with HBSS. Fluorescent intensities were measured at 0, 60 and 120 min in HBSS after the last HBSS wash at 485 nm (excitation)/528 nm (emission). The data were presented as relative fluorescence unit (RFU) for each well read by plate reader. For instance, medium control or vehicle control had a basal reading number in RFU, the basal level

of vehicle control at 60 min reading was around 1000 (Figure 3-2 A). When data were normalized with vehicle control, data were showed as RFU-Control in the figures.

Results and Conclusions

Dieldrin Induces a Dose-Dependent Production of ROS

To determine the effect of dieldrin on ROS production in microglia, we first treated rat HAPI microglial cells with vehicle ethanol (0.01% final concentration) or 1 μM dieldrin for 24 h. Afterwards, cells were loaded for 1 h with 2′,7′-dichlorofluorescin diacetate (DCF-DA) and fluorescent intensity, indicative of the amount of ROS produced, was determined at 0, 60 and 120 min after DCF loading. As shown in Figure 3-2 A, the fluorescent intensities of HAPI cells treated with 1 μM dieldrin for 24 h were significantly stronger than that of the vehicle-treated control cells at corresponding time points. The rate of increase in fluorescent intensity between 0 and 60 min after DCF loading for both the vehicle- and dieldrin-treated HAPI cells was markedly faster than that between 60 and 120 min. Therefore, for the subsequent studies, we chose to report the fluorescent intensities measured at 60 min after DCF loading. Next, we treated HAPI cells for 24 h with vehicle (0.01% ethanol) or 0.0001 to 1 μM dieldrin and measured ROS production using DCF-DA. As shown in Figure 3-2 B, treatment with dieldrin resulted in a concentration-dependent production of ROS in HAPI cells. When compared to cells treated with 0.0001 μM dieldrin, significant ROS production was observed in cells treated with 0.01, 0.1 or 1 μM dieldrin (Figure 3-2 B). To rule out the possibility that the increased fluorescent intensities observed in the dieldrin-treated HAPI cells were due to increased cell proliferation during the treatment period (24 h), we determined the total protein content of the control and dieldrin-treated cells. As shown in Figure 3-3, the total protein content of HAPI cells treated with 0.1–1 μM dieldrin was not significantly different from that of control cells,

suggesting that treatment with 0.1–1 μM dieldrin did not enhance the growth of HAPI cells and that the increase in fluorescent intensity was due to dieldrin-stimulated ROS production.

Time Course for Dieldrin-Induced ROS Production

HAPI cells were treated with vehicle control (0.01% ethanol), 0.01 or 1 μM dieldrin for 6, 12 or 24 h and ROS production was determined. No significant ROS production was detected in cells treated for 6 h with 0.01 or 1 μM dieldrin (Figure 3-4). However, by 12 h after stimulation, significant ROS production was observed in cells treated with 0.01 or 1 μM dieldrin. The dieldrin-induced ROS production continued to increase between 12 and 24 h after stimulation and the amount of ROS production at 24 h was significantly greater than that at 12 h for cells treated with either 0.01 or 1 μM dieldrin (Figure 3-4).

Effects of Dieldrin on ROS Production in Primary Rat Microglia and Astroglia

Primary rat microglia or astroglia were treated for 24 h with 0.001–1 μM dieldrin and ROS production was determined. Dieldrin induced a dose-dependent production of ROS in primary rat microglia and significant ROS production was observed in microglia treated with 0.1 and 1 μM dieldrin (Figure 3-5). The amount of ROS production in microglia treated with 0.01 μM dieldrin was greater than that for cells treated with 0.001 μM dieldrin. However, the differences were not statistically different. In primary rat astroglia, in contrast, significant ROS production was not observed following dieldrin treatment (Figure 3-5).

Effects of Dieldrin on ROS Production in Mouse BV2 Microglial Cells

In mouse BV2 microglial cells treated with dieldrin, a concentration-dependent ROS production was observed similar to that of rat HAPI microglial cells. Compared to BV2 cells treated with 0.001 μM dieldrin. Significant ROS production was observed in cells treated for 24 h with 0.1 or 1 μM dieldrin (Figure 3-6).

Discussion

Studies of various existing animal models of PD and postmortem human PD brains strongly suggest that microglial activation is intimately associated with dopaminergic neurodegeneration in the pathogenesis of PD (McGeer and McGeer, 2004; Tieu et al., 2003; Whitton, 2007). Activated microglia release a multitude of neurotoxic factors such as ROS that impact the oxidative damage-prone dopaminergic neurons to induce their eventual demise. In this study, we have demonstrated that nanomolar concentrations (10–1000 nM) of dieldrin are able to significantly augment ROS production in microglia (Figure 3-2, Figure 3-5, Figure 3-6). This finding may bear particular relevance to the potential effects on the nigrostriatal dopaminergic pathway in relation to the development of PD of chronic environmental exposure to low doses of dieldrin, a highly persistent and bio-accumulative OCP. Although the exact levels of dieldrin exposure in humans are difficult to quantify, levels of dieldrin found in the substantial nigra and striata of postmortem PD brains were as high as 0.515–0.924 μg/g lipid (Corrigan et al., 1998; Corrigan et al., 2000). These levels of residual dieldrin detected in the terminal stage of PD brains strongly implicate the possibility that microglia as well as nigra dopamine neurons in the brains of the affected individuals had at some point been exposed to medium to high nanomolar quantities of dieldrin. Microglia appear to be more responsive than neurons or astroglia to the presence of toxicants and the resultant microglial activation often results in increased neurotoxicity to dopaminergic neurons as illustrated in the studies of rotenone-induced dopaminergic neurotoxicity (Gao et al., 2002). Therefore, our findings in this study that microglial activation measured as increased ROS production is inducible by nanomolar concentrations of dieldrin suggest that potentially environmentally relevant levels of dieldrin may contribute to dopaminergic neurodegeneration in PD through the activation of microglia and the production of ROS.

Of the various microglia-released neurotoxic factors that include cytokines, nitric oxide, lipid metabolites, and oxygen free radicals, ROS may be the key contributor to dopaminergic neurodegeneration in PD. The innate characteristics of the nigrostriatal dopaminergic pathway may be the major underpinning of the heightened susceptibility of dopamine neurons to oxidative stress (Jenner and Olanow, 1998). These include reduced anti-oxidant capacity, high content of dopamine and lipids that are prone to oxidation, high content of iron that may promote the formation of more damaging free radicals through the Fenton reaction, and potential defect in mitochondrial complex I that may lead to elevated ROS generation (Greenamyre and Hastings, 2004; Youdim and Riederer, 1993). Furthermore, compared to other areas in the brain, the midbrain region that encompasses the nigrostriatal dopaminergic pathway may also be particularly abundant with microglia (Kim et al., 2000; Lawson et al., 1990). Therefore, although other factors should not be excluded, ROS from activated microglia, as observed in this study, clearly may play an important role in the dieldrin-induced dopaminergic neurodegeneration.

Although the precise underlying mechanisms of action remain to be delineated, our findings in brain immune cells (i.e., microglia) are in line with the reported effects of dieldrin on free radical generation in peripheral immune cells such as neutrophils (Hewett and Roth, 1988; Pelletier and Girard, 2002). It should be noted that the increased free radical generation in neutrophils in these studies was observed following short-term (< 2 h) treatment with micromolar concentrations of dieldrin. The increased ROS production observed in microglia in the current study, however, was a result of longer time (> 6 h and up to 12–24 h) induction with nanomolar concentrations of dieldrin (Figure 3-4). Identification of the inducible factors that contribute to the dieldrin-induced ROS production in microglia will certainly provide further insight into the related mechanisms of action. Nevertheless, increased microglial ROS

production observed for dieldrin may be proven, through additional in vitro and in vivo studies, to be reminiscent of the involvement of microglial NADPH oxidase in dopaminergic neurodegeneration induced by pesticide rotenone, neurotoxin MPTP, and bacterial endotoxin lipopolysaccharide (Gao et al., 2002; Qin et al., 2004; Wu et al., 2003).

Besides microglia, astroglia also play an important role in maintaining the homeostasis in the brain and providing trophic support to neurons (Aloisi, 1999). Astroglia also participate in mediating the neuro-inflammatory process through the production of nitric oxide and certain cytokines such as interleukin-6 (Farina et al., 2007). In this study, dieldrin-induced ROS generation was observed in rat and mouse microglia (Figure 3-2, Figure 3-6). In contrast, in primary rat astroglia treated with dieldrin under the same conditions as that for primary rat microglia (0.001–1 μM dieldrin for 24 h), significant rise in ROS production was not observed (Figure 3-5). Dieldrin was reported to be capable of inhibiting the gap junction communications of rat astroglia in culture and to potentiate the MPTP-induced reactive astrogliosis in developmentally dosed mice (Richardson et al., 2006). Whether this lack of response in dieldrin-stimulated ROS production in rat astroglia is a unique phenomenon or rather a reflection of the characteristically different spectrum of responses exhibited by astroglia and microglia (Liu and Hong, 2003) remains to be determined.

In summary, in this study, we, for the first time, have discovered that potentially environmentally relevant concentrations of the highly persistent and bio-accumulative organochlorinated pesticide dieldrin induce murine microglia to produce oxygen free radicals. This increased production of ROS may contribute to the degeneration of dopaminergic neurons in relevance to the environmental toxicant-related etiology of idiopathic PD.

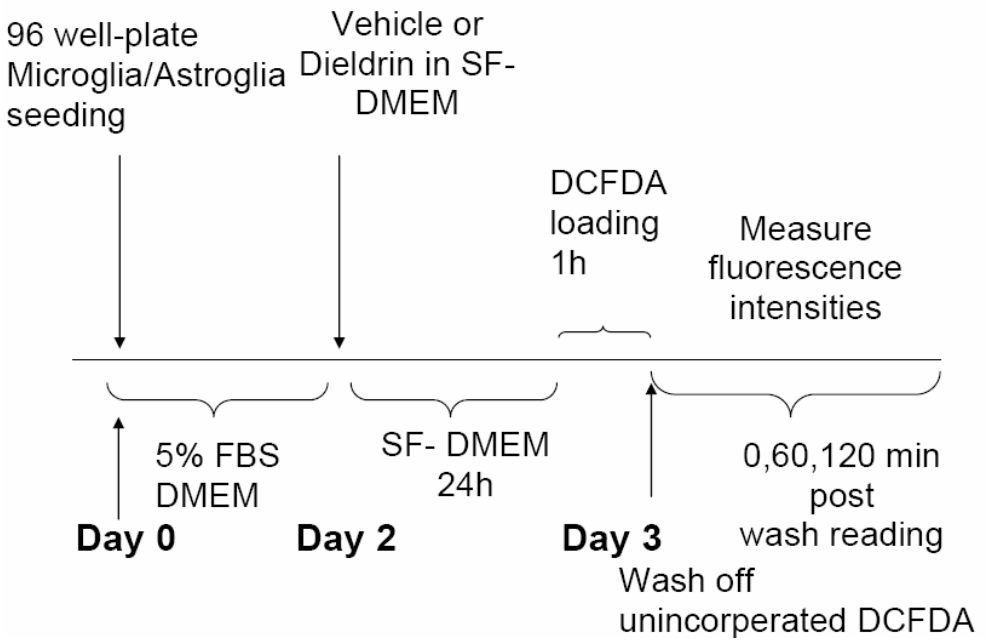
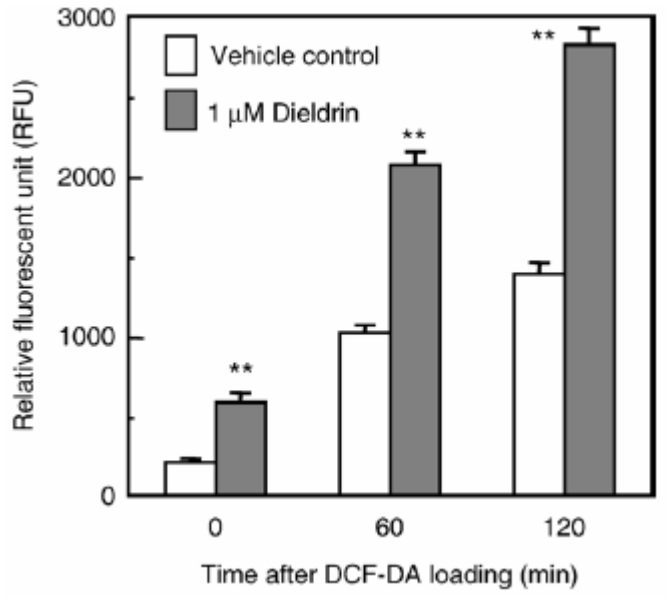
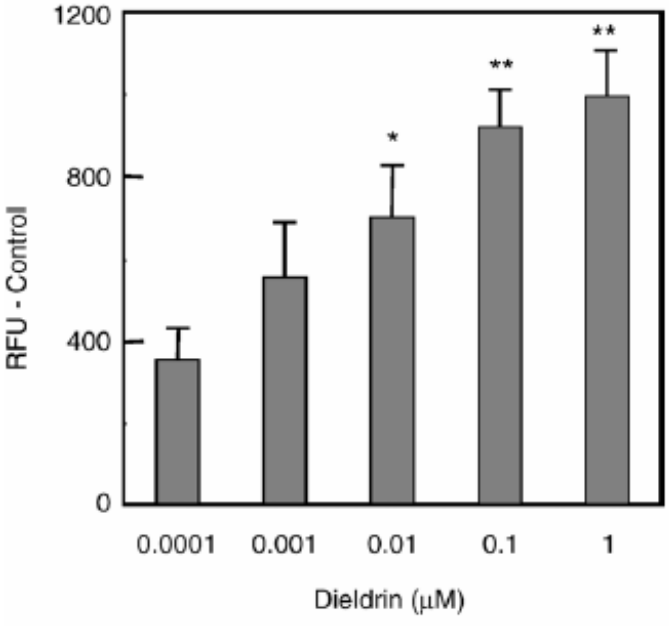


Figure 3-1. Flowchart of experimental design in aim one. DCF assay was used to detect ROS generation in microglia and astroglia in 96 well-plate. With 48 hours growth in serum medium, vehicle or dieldrin in series of concentrations treated the cells in serum-free medium for 24 hours. After treatment, DCF-DA was used as a non-selective fluorescent probe to detect several subtypes of ROS generation by 1 hour loading. Data showed as relative fluorescence unit (RFU) with reading at 0, 60 and 120min in HBSS.



A



B

Figure 3-2. Effect of dieldrin on ROS production in rat HAPI cells. A) HAPI cells were applied with vehicle control (0.01% ethanol) or 1 μM dieldrin for 24 h treatment. Generation of ROS was detected by DCF-DA (10 μM; 1h loading time). Fluorescent intensities shown to relative fluorescent units (RFU) were read at 0, 60, 120 min after DCF-DA 1h loading. Data refer to mean ± SEM of RFU values in one representative experiment (n=6 in each experiment). ** $p<0.005$ compared to time-matched vehicle controls. B) Hapi cells were treated for 24h with vehicle control (0.01% ethanol) or indicated concentrations of dieldrin and ROS generation was determined using DCF-DA. Data are measured as RFU values minus that of vehicle controls and shown as mean ± SEM with 6 experiments (n=6 in each experiment).

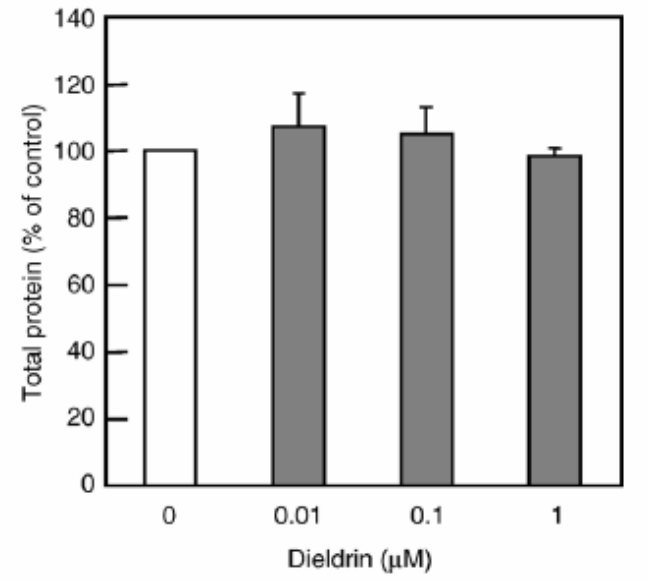


Figure 3-3. Total protein content of control and dieldrin-treated cells. Following treatment for 24 h with vehicle (0.01% ethanol) or indicated concentrations of dieldrin, total protein was determined as described in Experimental procedures. Results are expressed as a percentage of the control cells and are mean ± SEM of three experiments performed in triplicate.

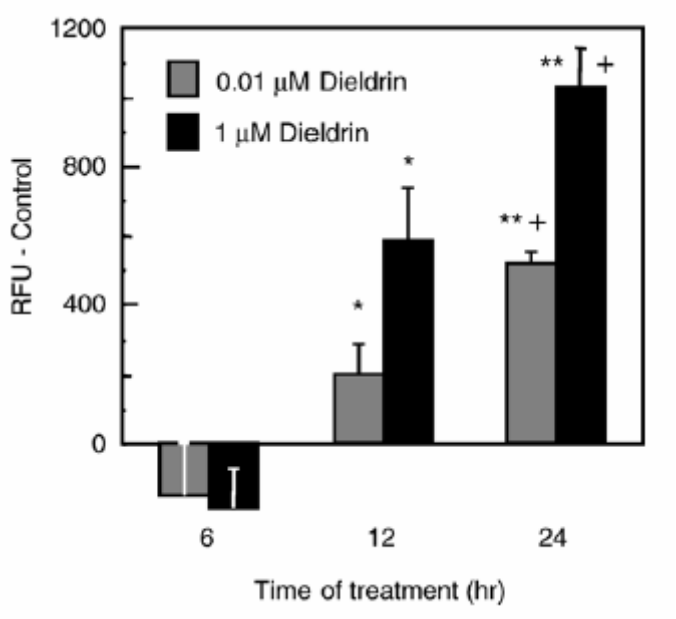


Figure 3-4. Time dependence of dieldrin-stimulated ROS production in rat HAPI microglial cells. Cells were treated with vehicle control (0.01% ethanol) or indicated concentrations of dieldrin for indicated timed intervals. Afterwards, ROS production was measured. Results are expressed as RFU minus time-matched controls and are mean ± SEM of 3–4 experiments performed in quadruplicate. * $p<0.05$ and **$p<0.005$ compared to corresponding groups at the 6-h time point; +$p<0.05$ compared to corresponding groups at the 12-h time point.

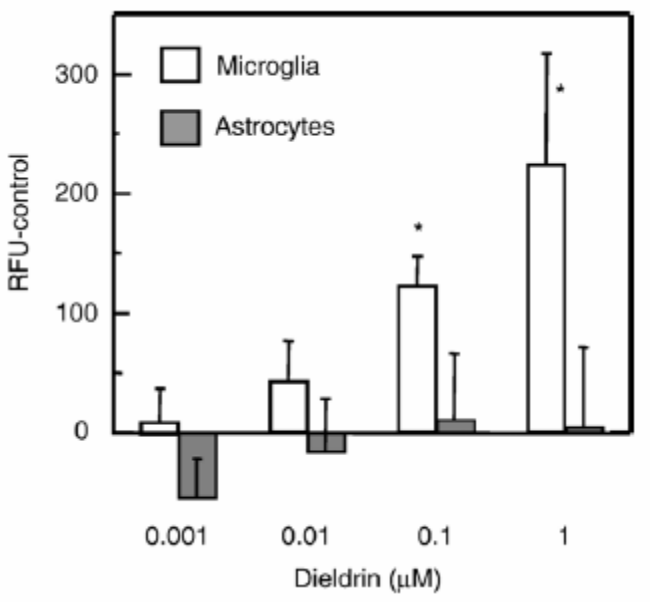


Figure 3-5. Effect of dieldrin on ROS production in rat primary microglia and astroglia. Cells were treated for 24 h with vehicle control (0.01% ethanol) or indicated concentrations of dieldrin. Production of ROS was measured using DCF-DA. Results are expressed as RFU minus vehicle-treated controls and are mean ± SEM of 3 and 4 experiments performed in sextuplicate for microglia and astroglia respectively. * $p<0.05$ and ** $p<0.005$ compared to cells treated with 0.001μM dieldrin.

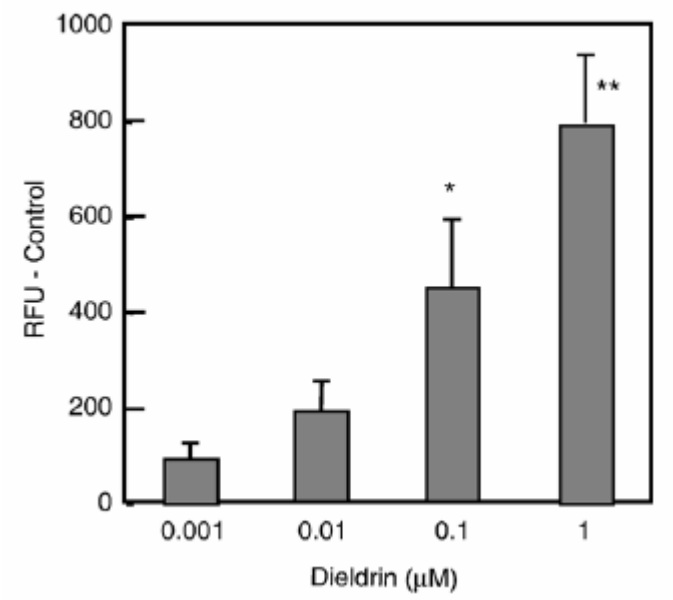


Figure 3-6. Effect of dieldrin on ROS production in mouse BV2 microglial cells. BV2 cells were treated for 24 h with vehicle control (0.01% ethanol) or indicated concentrations of dieldrin. Results are mean ± SEM of 5–6 experiments performed in sextuplicate. * $p<0.05$ and ** $p<0.005$ compared to cells treated with 0.001 μ M dieldrin.

CHAPTER 4
AIM TWO: COOPERATIVE EFFECT OF OCPS ON MICROGLIAL ROS GENERATION-NANOMOLAR COMBINATIONS OF LINDANE AND DIELDRIN

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder resulting from a massive degeneration of the movement-regulating nigrostriatal dopaminergic (DA) pathway (Langston, 2002). Although the cause(s) of idiopathic PD remains unclear, environmental exposure to pesticides has been proposed to play an important role in the development of PD (Kamel et al., 2007; Liu et al., 2003a; Liu and Hong, 2003). Animal studies have shown that administration of pesticides paraquat/maneb and rotenone induce degeneration of nigral DA neurons and reproduces behavioral deficits of PD (Cicchetti et al., 2005; Thiruchelvam et al., 2000).

The organochlorinated pesticides (OCPs) were among the most widely and heavily used pesticides from the 1940s to 1990s. Large-scale agricultural applications of OCPs were curtailed starting in the 1970s and eventually stopped in the 1990s (Kanthasamy et al., 2005; Luzardo et al., 2006). However, the sheer quantities of OCPs used, combined with their extreme persistence in the environment and bioaccumulation along the food chain have resulted in the presence of high levels of residual OCPs in the soil, water, and our daily food supplies (Hickey et al., 2006; Schafer and Kegley, 2002). Significant levels of dieldrin and lindane have been detected in the sera of human populations around the world (Bates et al., 2004; Botella et al., 2004). In addition, a number of studies have found several OCPs including dieldrin and lindane to be enriched in the substantia nigra and striata of certain postmortem PD brains compared to age-matched control brains (Corrigan et al., 2000; Fleming et al., 1994). Furthermore, case-control studies have repeatedly associated exposure to OCPs with increased risk of developing PD (Hancock et al., 2008; Seidler et al., 1996). Experimentally, dieldrin has been reported to induce a caspase-

mediated degeneration of DA neurons in cultures (Kitazawa et al., 2003; Sanchez-Ramos et al., 1998). Perinatal administration of dieldrin in mice augmented the susceptibility of the nigrostriatal DA pathway of the offspring to insult by other DA neurotoxins (Richardson et al., 2006). Furthermore, dieldrin administration induced significant oxidative damage to the nigrostriatal DA pathway (Hatcher et al., 2007). The potential effect of lindane on DA neurons, especially those associated with low dose environmental exposure, however remains largely unknown.

The nigrostriatal DA neurons are known to be highly vulnerable to oxidative damage (Greenamyre et al., 1999). One of the major contributors of oxidative stress is the activation of the resident brain immune cells, microglia that are particularly abundant in the midbrain region (Liu, 2006). A variety of neurotoxicants have been shown to activate microglia to release reactive oxygen species (ROS), as well as cytokines and lipid metabolites that exacerbate DA neurodegeneration. In this study, in light of the co-existence of residual OCP mixtures in the environment, food supplies and certain postmortem PD brains, we set out to determine the effects of combinations of lindane and dieldrin on ROS generation in microglia.

Methods

Experimental Design Rationale

With the results from aim one, both rat and mouse microglial cell lines were shown to be able to produce increased ROS with dieldrin induction. Moreover, either MPTP model or neurodegeneration model was effective in mouse model. Therefore mouse BV2 microglial cells were used to study the cooperative effect of dieldrin and lindane combinations.

In the first aim, ROS generation was determined by non-selective fluorescent probe DCF-DA in different cell culture of microglia. Here as second step, with the treatment of OCP

combinations, detection of specific ROS subtype would better demonstrate the effect of OCPs on microglia. Moreover, although DCF-DA is able to detect most subtypes of ROS intracellularly, the superoxide anion is not one of them. As we know, superoxide anion is a very important source of oxidative stress (Muller et al., 2006), it would be reasonable to detect specific superoxide anion production in microglia. Therefore, selective fluorescent probe DHE was used in second aim as an indicator of superoxide production in microglia.

Many OCPs have accumulated in the environment over time. The combination of two OCPs could be a simplified exposure model to investigate the risk factor of environmental OCP exposure. Dieldrin and lindane were selected as candidates, because of the coexistance of these two OCPs in the same samples from normal populations and from post-mortem PD brains which exhibit elevated levels (Bates et al., 2004; Corrigan et al., 2000). As second step, in second aim, ROS generation induced by two OCPs dieldrin and lindane were studied in simplified combinations to represent the pesticide exposure in the environment. Here the hypothesis was to determine if combinations of dieldrin and lindane induced ROS generation in a synergistic manner. Before the experimental design used, for the combinations, single OCP dieldrin or lindane induced superoxide production was detected to determine the optimal concentrations for the cooperative study. In order to detect the cooperative effect of combinations, the ideal concentrations of dieldrin or lindane should be in the low nanamolar levels which individually only cause a slight increases on ROS generation.

Treatment

For each treatment, the stock solutions (10 mM) of dieldrin or lindane were freshly prepared in absolute ethanol. Rat microglial BV2 cells were prepared and seeded into 96-well plate on day 0 in the DMEM with 5% FBS, at 4×10^4 /well cell density with 100 μL/well medium at 37°C and 5% CO_2 (Figure 4-1). 48 hours later, the cells reached 90% confluence in wells.

After 48 hours growing in the serum medium for confluence, in order to decrease the influence from other factors in the serum such as growth factors, cells were switched to and maintained for 1 h at 37°C and 5% CO_2 in serum-free and phenol red-free DMEM (treatment medium). Afterwards, cells adapted with the treatment medium were treated (6 wells/condition) at 37°C and 5% CO_2 for desired timed intervals (24 hours) with vehicle control (0.01% ethanol in medium), or dieldrin or lindane alone at 1, 10 and 100 nM concentrations, or four combinations of dieldrin and lindane at 1 or 10 nM concentrations in the treatment medium. For DHE assay, light-sensitive DHE at 20 μM was loaded for 30 min and washed off with HBSS. Fluorescent intensities were measured at 0, 10 and 30 min in HBSS after the last HBSS wash at 520 nm (excitation)/590 nm (emission). Data were presented in relative fluorescence unit (RFU) for each well read by plate reader. When the data were normalized with vehicle control, data were presented as RFU-Control in the figures. For DCF assay, it is similar to the experimental design in aim one (Figure 3-1), while the treatment of DCF assay in aim two is groups in vehicle control, or dieldrin or lindane alone, or dieldrin and lindane combination.

Results and Conclusions

Single OCP Induced ROS Generation in BV2

OCPs were thought of as risk factors in PD etiology with their wide use and large amount in the environment (Liu et al., 2003a; Luzardo et al., 2006; Schafer and Kegley, 2002). OCP member lindane and dieldrin were tested separately on ROS production in activated mouse BV2 microglial cells. We treated BV2 cells with vehicle control (0.01% ethanol) or 1 to 100 nM lindane or 1 to 100 nM dieldrin for 24 h and determined ROS production by DHE at 20μM. In Figure 4-2 A, Lindane at 1-100 nM induced significant ROS production above the vehicle-treated control cells. When compared to 1 nM lindane-treated cells, we only found 10 nM lindane-treated cells had significant ROS generation (Figure 4-2 A). In Fig. 4-2 B, significant

ROS production was also found in BV2 cells treated with 1 nM to 100 nM dieldrin compared with vehicle control cells. Similar to that of lindane, only 10 nM dieldrin-treated cells had significant ROS generation compared with that of 1 nM dieldrin-treated groups (Figure 4-2 B). This ROS production from dieldrin-treated cells is in accordance with what we found in the previous study and may be related to neuroinflammation in PD (McGeer and McGeer, 2004).

Synergistic Effects of OCP Combinations with Lindane and Dieldrin on ROS Generation

Lindane or dieldrin demonstrated the same pattern of ROS production in microglia with DHE assay in Figure 4-2, since that combination of OCPs has been found in a epidemiological study (Luzardo et al., 2006). Is it possible that the mixture of these two OCP will lead to a synergistic pattern of ROS production in microglia? In order to investigate the ability of OCP combination, cells were treated with four combinations of 1 or 10 nM lindane mixed with 1 or 10 nM dieldrin. Cells treated with these four mixtures have elevated ROS generation compared with ROS generated from four additions of lindane-treated cells and dieldrin-treated cells respectively (Figure 4-3). The combination of 1 nM lindane and I nM dieldrin was most effective in inducing a synergistic increase in microglial ROS that was more than three times the sum of that in cells treated with 1 nM lindane and 1 nM dieldrin alone (Figure 4-3 A). The combination of 1 nM lindane and 10 nM dieldrin, 10 nM lindane and 1 nM dieldrin, or 10 nM lindane and 10 nM dieldrin were significantly more potent than either agent alone at the corresponding concentrations (Figure 4-3 B, C, D). Moreover, the levels of ROS generation from cells treated with lindane/dieldrin mixture groups were significantly greater than that of the respective lindane alone group or dieldrin alone group (Figure 4-3). Taken together, we demonstrated synergistic ROS production effects of nanomolar levels lindane and dieldrin mixture in BV2 microglial cells.

Not only using the DHE assay, but intracellular ROS generation detected by DCF assay showed similar trends of a synergistic effect with treatment of the combination of lindane and dieldrin at 10 nM (Figure 4-4).

In conclusion, the results from our study demonstrate that both lindane and dieldrin can induce ROS production in microglial cells. Furthermore, a synergistic effect of low nanomolar levels of lindane and dieldrin mixtures has been found in ROS generation. This is the first time that the combination of two OCPs have been shown to induce microglia to express increased ROS, and this OCP-induced increase in ROS production may be involved as an environmental risk factor in sporadic PD cases.

Discussion

Activation of microglia and production of ROS are known to contribute to the degeneration the oxidative damage-prone DA neurons in the development of PD (Liu, 2006). In this study, treatment of mouse BV2 microglia for 24 hr with nanomolar concentrations of either lindane or dieldrin alone resulted in a concentration-dependent production of ROS measured as the oxidation of DHE (Figure 4-2). To determine the combined effects of lindane and dieldrin on microglial ROS generation, we treated BV2 cells for 24 hr with combinations of 1 or 10 nM of lindane with 1 or 10 nM of dieldrin. As shown in Figure 4-3, all four combinations of lindane and dieldrin were significantly more potent in inducing microglial ROS generation than individual agents used alone. However, with increasing concentrations of lindane and/or dieldrin, the differences between the amount of ROS generation induced by the combinations and the sum of that induced by individual agents decreased accordingly (Figure 4-3 B, C and D). No cooperative ROS generation was observed with the combination of 100 nM of lindane and 100 nM of dieldrin (data not shown). All the final RFU numbers of four combinations normalized with vehicle control were in the same level (around 250) and it would be a ceiling effect. A

ceiling effect of OCP combination means that with increased doses of dieldrin or lindane, the combination have a smaller increase of ROS production. These results demonstrated that lindane and dieldrin, especially at low nanomolar concentrations were capable of synergistically stimulating microglia to generate ROS. Since DA neurons are highly susceptible to oxidative damage, lindane and dieldrin-induced synergistic ROS generation may contribute to the induction of oxidative stress and ultimately DA neurodegeneration in animal PD models and humans (Hatcher et al., 2007; Greenamyre et al., 1999).

Results in this study show that two widely present and highly persistent OCPs, lindane and dieldrin, at low nanomolar concentrations synergistically activate microglia to produce ROS through a potentially NADPH oxidase-mediated mechanism. In light of vulnerability of nigrostriatal DA pathway to oxidative stress, lindane and dieldrin-induced synergistic microglial ROS production may be an important contributor to the pesticide-induced DA neurodegeneration. Furthermore, the synergism of OCP in activating brain immune cells may be highly relevant to the multi-factorial etiology for the development of sporadic PD.

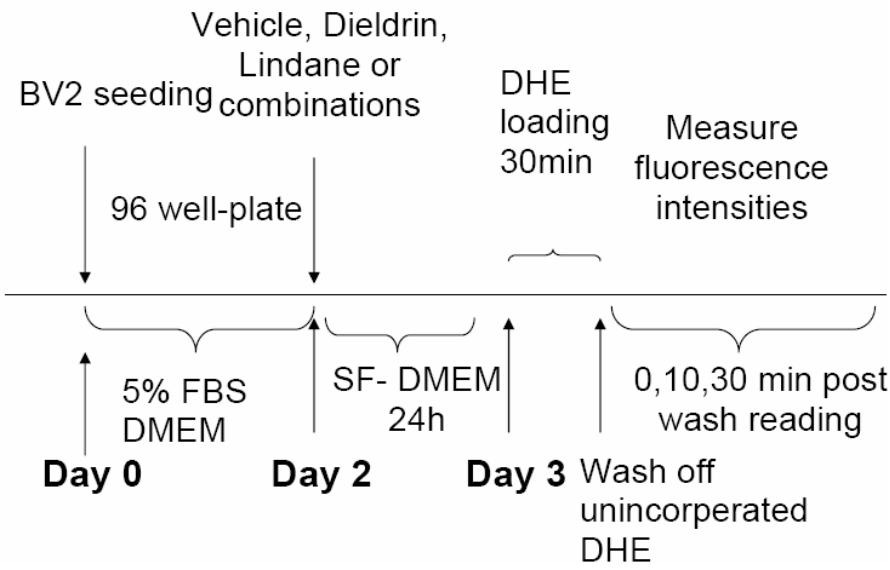
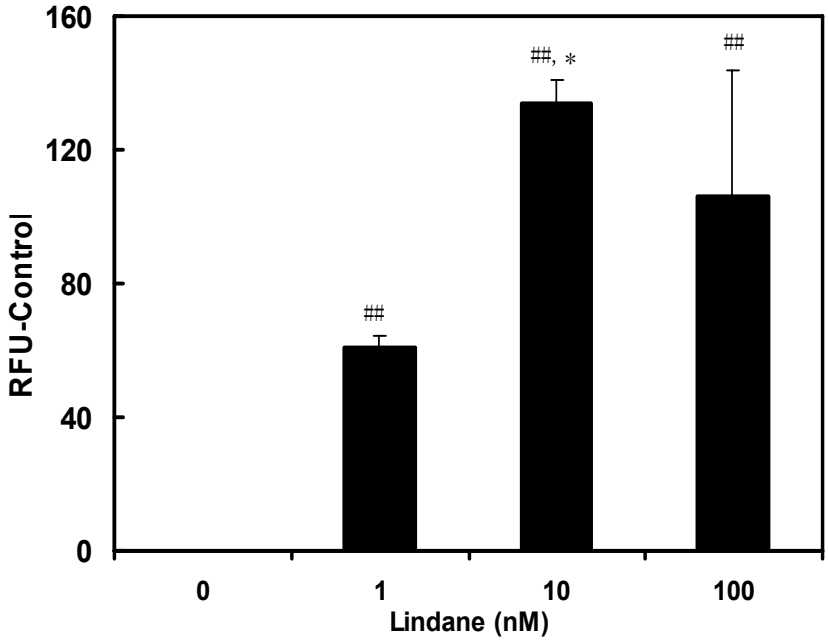
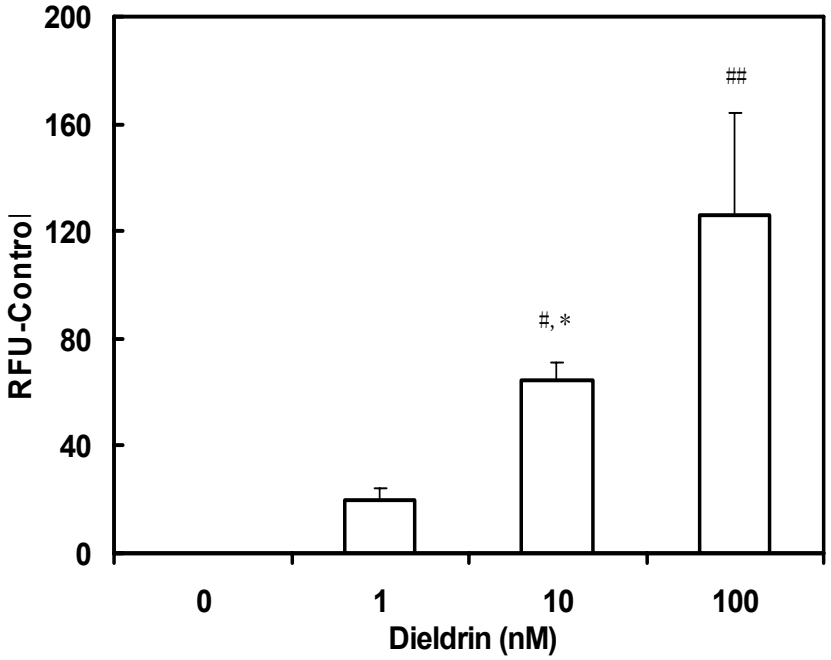


Figure 4-1. Flowchart of experimental design in aim two. DHE assay was used to detect superoxide anion generation in mouse microglial cell line BV2 in 96 well-plate. With 48 hours growth in serum medium, vehicle or dieldrin or lindane or mixture of dieldrin and lindane at 1 or 10 nM concentrations treated BV2 cells in serum-free medium for 24 hours. After treatment, DHE was used as a selective fluorescent probe to detect specific superoxide anion generation by 30 min loading. Data showed as relative fluorescence unit (RFU) with reading at 0, 10 and 30min in HBSS.



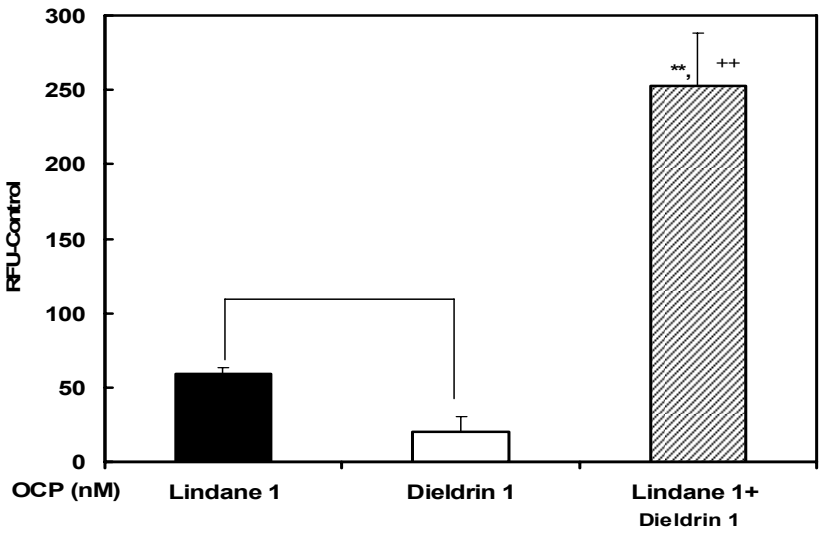
A

Figure 4-2. Effect of OCP on ROS generation in BV2 cells detected by DHE assay. Cells were treated with vehicle control (0.01% ethanol) or lindane A) at 1, 10, 100nM or dieldrin B) at 1, 10, 100nM for 24h. Fluorescent intensities data illustrated as relative fluorescent units (RFU) values minus that of vehicle controls. A) Results are expressed as mean ± SEM of four or six experiments (n=6 in each experiment). B) Results are expressed as mean ± SEM of three or five experiments (n=6 in each experiment). * $p<0.05$ compared with 1nM OCP groups. # $p<0.05$ and ## $p<0.005$ compared with corresponding controls.



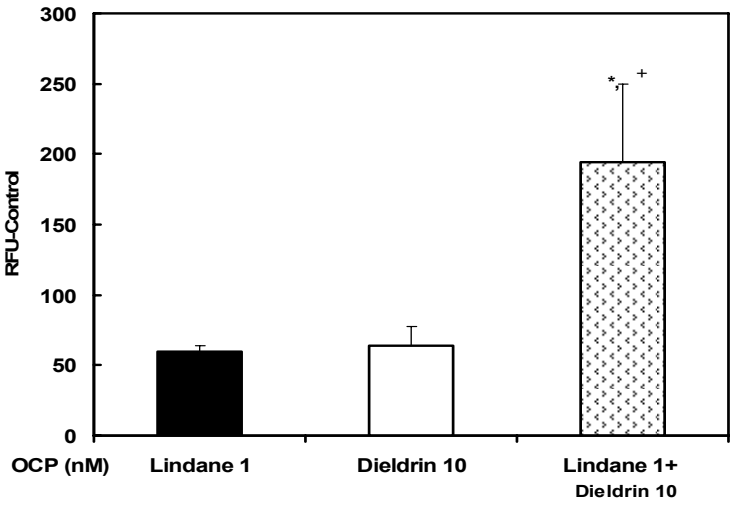
B

Figure 4-2. Continued

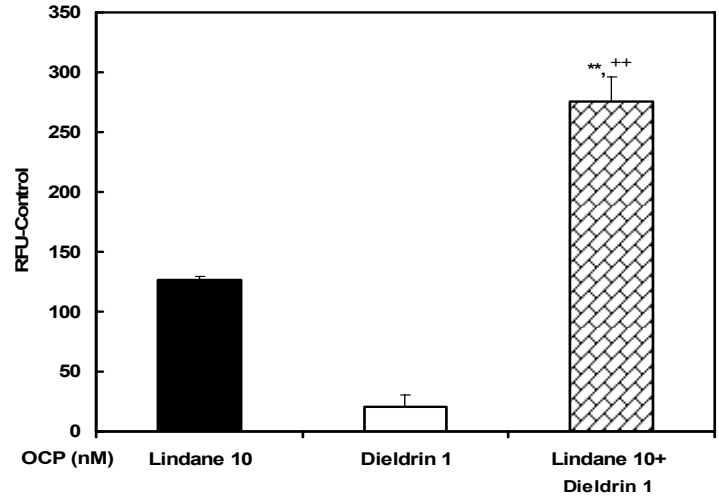


A

Figure 4-3. Synergistic superoxide generation induced by OCP mixture (lindane/dieldrin) in BV2 cells detecting by DHE assay. Results are expressed as RFU values minus that of vehicle controls. A) Cells were treated with lindane at 1nM or dieldrin at 1 nM or 1 nM lindane/1 nM dieldrin mixture for 24 h. B) Cells were treated with lindane at 1nM or dieldrin at 10 nM or 1 nM lindane/10 nM dieldrin mixture for 24 h. C) Cells were treated with lindane at 10 nM or dieldrin at 1 nM or 10 nM lindane/1 nM dieldrin mixture for 24 h. D) Cells were treated with lindane at 10 nM or dieldrin at 10 nM or 10 nM lindane/10 nM dieldrin mixture for 24 h. Data are showed as mean ± SEM of five experiments (n=6 in each experiment). * $p<0.05$ and ** $p<0.005$ showed lindane/dieldrin mixture groups compared with lindane alone groups. + $p<0.05$ and ++ $p<0.005$ showed lindane/dieldrin mixture groups compared with dieldrin alone groups.



B



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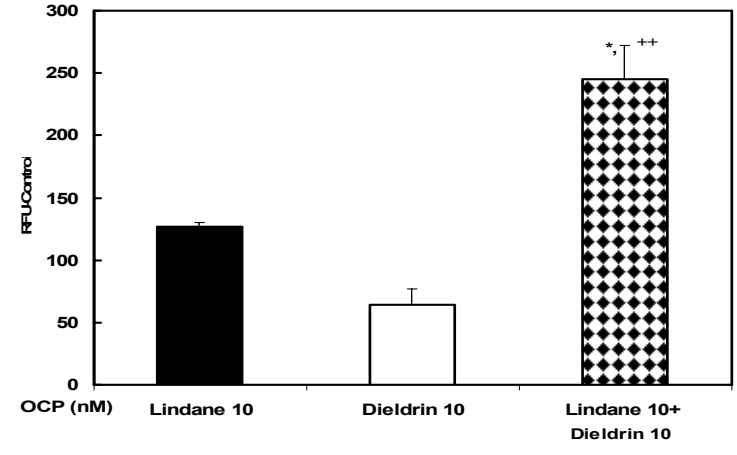


D

Figure 4-3. Continued

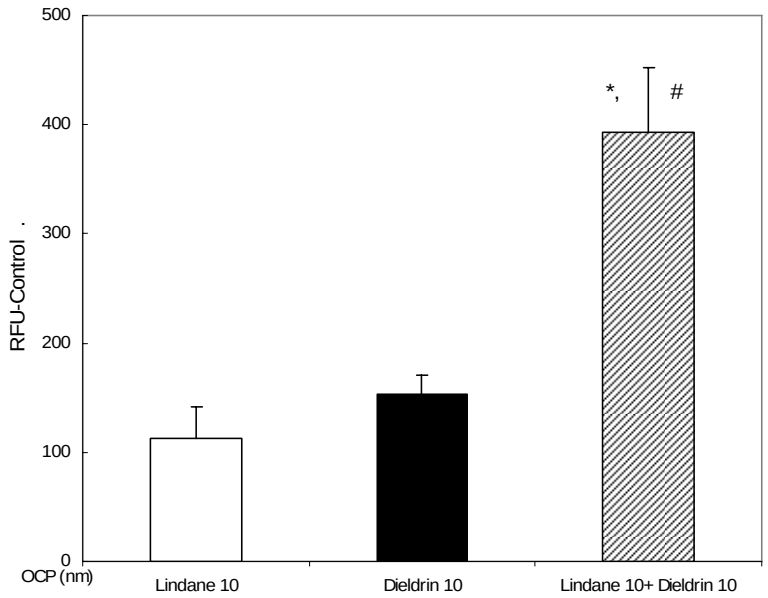


Figure 4-4. Synergistic ROS generation induced by OCP mixture (lindane/dieldrin) in mouse microglial BV2 cells detecting by DCF assay. Cells were treated with lindane at 10 nM or dieldrin at 10 nM or 10 nM lindane/10 nM dieldrin mixture for 24 h. Data are showed as mean ± SEM of five experiments (n=6 in each experiment). * $p<0.05$ showed lindane/dieldrin mixture groups compared with lindane alone groups. # $p<0.05$ showed lindane/dieldrin mixture groups compared with dieldrin alone groups.

CHAPTER 5
AIM THREE: MECHANISMS OF ACTION FOR OCP-INDUCED MICROGLIAL ROS GENERATION: INVOLVEMENT OF NADPH OXIDASE AND GABA RECEPTORS

Introduction

In the previous two aims, the important role of activated microglia was demonstrated with OCP-induced ROS production both in single exposure and combination exposure. Subsequent question raised from this finding is: what are the underlying mechanisms of ROS generation in activated microglia after OCP exposure. Increasing evidence supports the idea that NADPH oxidase may play a role in activated microglia mediated ROS generation. Several well-established PD models, including MPTP model, 6-OHDA model and LPS model, demonstrated involvement of NADPH oxidase with evidence of elevated expression NADPH oxidase subunits or inhibitory effects of NADPH oxidase inhibitors (Qin et al., 2004; Rodriguez-Pallares et al., 2007; Wu et al., 2003). Therefore, inhibition of microglial ROS generation by pharmacologic inhibitors or gene deletion of NADPH oxidase has been found to protect dopamine neurons from toxin-induced degeneration (Liu et al., 2000; Qin et al., 2004; Wu et al., 2003). In this study, specific inhibitors of NADPH oxidase were used to study the potential underlying mechanisms of NADPH oxidase-mediated ROS generation in microglia.

GABA is well-known as an inhibitory neurotransmitter in the CNS, and there are 3 types of GABA receptors distributed in neurons. At micromolar doses of dieldrin administration, it is known that dieldrin altered developmental expression of GABA receptor subunits in GABAergic neurons, resulting in altered functions of GABA receptor in the neuron (Liu et al., 1997). In cockroach neurons, dieldrin demonstrated a biphasic effect on GABA-induced current from GABA receptor as potentiation followed by inhibition, and this potentiation is more potent than that of inhibition in a dose-sensitive manner (Zhao et al., 2003).

Recently GABA receptors have been identified in non-neurons including astrocyte and microglia (Charles et al., 2003; Duke et al., 2004). Moreover, $GABA_B$ receptor mediated neutrophil-dependent inflammation in human stroke injury. These findings suggested that GABA receptors may contribute to neuroinflammation via microglial activation. In this study, GABA receptor agonists and antagonists were applied to study the role of GABA receptor in OCP-induced microglial activation. As for the signaling pathways for ROS generation, protein kinase C (PKC) was stimulated in inflammatory pathways relating to NADPH oxidase and GABA receptors (Kelly et al., 2002; Min et al., 2004; Song and Messing, 2005). Both ROS generation and cytokine release were detected in activated microglia via PKC activity. PKC is a potential co-mediator of NADPH oxidase or GABA receptor in the possible mechanisms of ROS generation in activated microglia.

In this part, the underlying mechanisms of OCP-induced ROS production in activated microglia will be studied by investigating the role of NADPH oxidase, GABA receptors and PKC activity in the process of ROS generation.

Methods

Experimental Design Rationale

With the results form first and second aims, OCP-induced increased ROS generation have been shown in different cell lines or primary microglia with non-selective and selective fluorescent probes. Based on this conclusion, the next step in the project was to understand the underlying mechanisms of OCP exposure induced ROS generation in microglia.

Increasing evidence supports the role of NADPH oxidase in the neuronal death during neurodegenerative diseases with ROS overproduction in the CNS (Infanger et al., 2006). For instance, in post-mortem brains of Alzheimer's disease patients, elevated cytosolic NADPH

oxidase is located in the damage region (Shimohama et al., 2000). Moreover, β-amyloid stimulates NADPH oxidase activation to promote apoptosis with excessive ROS (Wilkinson and Landreth, 2006). In cerebral ischemic injury, reduced brain injury was detected in gp91 ^{phox} knockout mice without functional NADPH oxidase activity (Walder et al., 1997). NADPH oxidase was shown to be involved in MPP^+-induced DA neuronal loss, and NADPH oxidase selective inhibitors DPI and apocynin attenuated the neuronal loss (Anantharam et al., 2007). Besides DA neurons, in PD study of rat 6-OHDA model, NADPH oxidase was shown to be activated in microglia. In the same study, treatment with NADPH oxidase activator angiotensin II had the opposite effect of apocynin (Rodriguez-Pallares et al., 2007). Therefore, NADPH oxidase was hypothesized to be a critical player in the OCP-induced ROS generation in microglia.

Two NADPH oxidase inhibitors with different selective inhibitory mechanisms were used in the experimental design. In Figure 5-1, the mechanisms of action of both NADPH oxidase and its inhibitors DPI and apocynin are presented. As an enzyme complex, NADPH oxidase has six components: membrane-bound cytochrome b_{558} consists of $p22^{phox}$ and gp91 ^{phox}, cytosolic $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and a Rho guanosine triphosphatase member, usually Rac1 or Rac2 (Babior, 1999). Activation of $p47^{phox}$ is the first step of NADPH oxidase functional generation of ROS in activated cell. After activation, $p47^{phox}$ will integrate with $p40^{phox}$ and $p67^{phox}$ to form the cytosolic part. With GTP binding of Rac, the assembly of $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ will bind with membrane-bound cytochrome b_{558} to form a functional NADPH oxidase complex, the complex will catalyze intracellular ROS and proton generation in neutrophil (Assari, 2006). Here the hypothesis was that NADPH oxidase catalyzed ROS generation in microglia via a similar way as that in neutrophils. In the presence of NADPH oxidase selective inhibitor DPI (Yu et al.,

2007), DPI inhibited NADPH oxidase activity via covalent binding to FAD (Figure 5-1). The other selective inhibitor apocynin (Muijsers et al., 2000), inhibits NADPH oxidase-catalyzed ROS generation by blocking the translocation of cytosolic assembly (Figure 5-1).

Dieldrin and lindane were related to $GABA_A$ receptor through the chloride-channel complex, and both stimulatory and inhibitory effects have been found in neurons (Nagata et al., 1994). In this aim, $GABA_A$ receptor was investigated with dieldrin-induced ROS in microglial cells. Selective $GABA_A$ antagonist picrotoxin (Das et al., 2003) was used to evaluate the $GABA_A$ receptor involvement in dieldrin-induced ROS generation in microglia.

In rat CNS, all three $GABA_B$ receptor subunits $GABA_BR1a$, $GABA_BR1b$ and $GABA_BR2$ were detected by immunohistochemistry in activated microglia culture (Charles et al., 2003). Furthermore, both activated microglia in culture and in tissue from rats expressed these three $GABA_B$ receptor subunits by western blotting. When activation of $GABA_B$ receptors occurred, cytokines IL-6 and IL-12p40 was attenuated in the LPS-induced microglia activation model (Duke et al., 2004; Kuhn et al., 2004), suggesting that $GABA_B$ receptors may also act as a mediator in neuroinflammation of microglia. Selective $GABA_B$ antagonist CGP 35348 has a potent antagonism on $GABA_B$ receptor (Olpe et al., 1990). In this aim, CGP 35348 was used to investigate the involvement of $GABA_B$ receptors in dieldrin-induced ROS generation in microglia.

Treatment

For each treatment, a stock solution (10 mM) of dieldrin or lindane was prepared fresh in absolute ethanol and diluted into final concentration in HBSS, and GABA was prepared fresh in HBSS. For treatment with DPI inhibitors, cells were switched to and maintained for 1 hour at 37°C and 5% CO_2 in serum-free and phenol red-free DMEM (treatment medium) after 48 hours growth in the plate with serum DMEM. Afterward, cells were treated (4–6 wells/condition) at

37°C and 5% CO_2 for 24 hours with vehicle control (0.01% ethanol) or desired concentrations of dieldrin or lindane diluted in the treatment medium. DPI or apocynin was prepared in absolute ethanol and diluted in serum-free medium, then added 18 hours after the beginning of OCP treatment. Cycloheximide or actinomycin D prepared in absolute ethanol and diluted in serum-free medium, then added 15 min before DCF loading. After 24 hours treatment with vehicle control or desired OCP, non-selective fluorescent probe DCF at 10 μM or selective fluorescent probe DHE at 20 μM was applied to detect ROS production with 1 hour or 30 min loading respectively. Afterwards, plates were read at desired time intervals.

For short-term (0-120 min) treatment on GABA receptors involvement, DCF assay was used to detect ROS generation in HAPI cells. In Figure 5-2, the diagram of short-term study showed the treatment with GABA antagonists and dieldrin or GABA. HAPI cells seeded into 96-well plate and grew for 48 hours with 90% confluence in DMEM with 5% FBS. DCF at 10 μM in HBSS was loaded for 1 hour first, then removed DCF solution, and cells was treated with dieldrin or GABA in HBSS solution for desired time intervals at 0, 60 and 120 min, each time point was detected for DCF assay. For the wells treated with $GABA_A$ antagonist picrotoxin, $GABA_B$ antagonist CGP35348 or PKC inhibitor staurosporine, reagents were fresh prepared in absolute ethanol and diluted into HBSS, then added 15 min before dieldrin or GABA treatment at desired concentrations.

Results and Conclusions

Effect of NADPH Cxidase Inhibitors on the Dieldrin-Induced ROS Production in HAPI Cells

Since NADPH oxidase is a major enzyme complex responsible for ROS production in immune cells including microglia (Babior, 2004), we used inhibitors of NADPH oxidase to determine the involvement of this enzyme complex in the dieldrin-induced ROS production in

microglia. As shown in Figure 5-3, dieldrin (1 μM; 24 h)-induced ROS production was significantly reduced by diphenylene iodonium (DPI) (Irani et al., 1997)or apocynin (Stolk et al., 1994), inhibitors of the catalytic activity of NADPH oxidase that interfere with the assembly of the enzyme complex. Treatment with DPI (0.1 and 0.5 μM) or apocynin (10 and 50 μM) alone did not significantly affect the basal ROS levels of the cells (Figure 5-3). The presence of vehicle control (0.0025% DMSO for DPI and 0.025% ethanol for apocynin) did not have any significant effects on either the basal or dieldrin-induced ROS production.

Effects of Inhibitors of Gene Transcription and Protein Synthesis on the Dieldrin-Induced ROS Production in HAPI Cells

The time-dependent induction by dieldrin of ROS production in HAPI cells (Figure 3-5) prompted us to determine the effects of inhibition of gene transcription or protein synthesis on the dieldrin-induced ROS production in HAPI cells. Cycloheximide was used to inhibit protein synthesis and actinomycin D to prevent gene transcription (Fulda et al., 2000; Sobell, 1985). Pretreatment of cells with cycloheximide or actinomycin D prior to treatment with dieldrin (1 μM; 12 h) dose dependently reduced dieldrin-induced ROS production (Figure 5-4). Treatment with cycloheximide or actinomycin D alone did not have any significant effect on the basal level of ROS production in HAPI cells.

Inhibitory Effect of NADPH Oxidase Inhibitor DPI in Rat Primary Microglia

Similar to that observed for rat HAPI microglial cells (Figure 5-3), the dieldrin-induced ROS production in rat primary microglia was inhibited by the NADPH oxidase inhibitor DPI (Figure 5-5). Treatment with DPI alone did not have a significant effect of the basal ROS generation in primary microglia (Figure 5-5).

Effect of NADPH Oxidase Inhibitor DPI in OCP Combination-Induced ROS Generation

As a main enzyme complex related to ROS production in microglia, NADPH oxidase (Babior, 2004) is involved in single OCP dieldrin induced ROS generation because of the inhibitory effects of DPI and apocynin observed in rat microglia (Figure 5-3, 5-5). Furthermore, DPI was used to determine the involvement of NADPH oxidase in ROS production in lindane and dieldrin treated mouse microglia BV2 cells. As illustrated in Figure 5-6, DPI significantly inhibited lindane and dieldrin-induced ROS generation stimulated by either 1nM dieldrin combined with 1nM lindane or 10 nM lindane (Figure 5-6A, B). DPI at 0.1 μM more effectively attenuated ROS generation in OCP induced by low nanomolar concentrations of lindane or dieldrin administration in microglia than that by micromolar concentration of dieldrin in rat microglia. Treatment of DPI at 0.1 or 0.5 μM alone didn't significantly influence basal ROS levels.

GABA Induced ROS Generation in HAPI Cells

Since GABA is a natural GABA receptor agonist, introducing GABA into microglia would be helpful to understand the role of GABA receptors in activated microglia. Using a short-term stimulation scheme, a concentration-dependent increase in ROS generation was detected in GABA-treated groups as well as that of dieldrin-treated microglia (Figure 5-7). Compared with lowest concentration, 50 μM GABA induced significant ROS generation, although it is not as effective as dieldrin at the same concentration. Since GABA is capable of inducing ROS generation in microglia, GABA may be a potential players involved in ROS generation.

Effect of GABA Antagonists on the Dieldrin-Induced ROS Production in HAPI Cells

With the evidence of GABA-induced ROS generation, GABA antagonists were used to determine if they antagonize dieldrin-induced ROS generation. In Figure 5-8 A, treatment with $GABA_B$-selective antagonist CGP 35348 significantly attenuated dieldrin-induced elevated

levels of ROS generation in concentration-dependent manner in HAPI cells. $GABA_B$ antagonist CGP 35348 at 1 μM reduced dieldrin-induced ROS production by half. At 10 μM CGP 35348, around 90% dieldrin-induced ROS production was inhibited compared with that of dieldrin alone. $GABA_A$ selective antagonist picrotoxin, reduced dieldrin-induced ROS generation in HAPI cells in similar manner as CGP 35348 (Figure 5-8. B). Picrotoxin at 0.1μM or 1 μM attenuated by the dieldrin-induced ROS generation 40% or 80% respectively. Based on these results, both CGP 35348 and picrotoxin effectively attenuated ROS release from activated microglia, suggesting that dieldrin-induced ROS generation may involve receptor activities both in $GABA_A$ and $GABA_B$ receptors.

Effect of PKC Activity on the Dieldrin-Induced ROS Production in HAPI Cells

In LPS-induced microglia activation, inhibition of PKC activity was effective in inhibiting free radical release, in activated primary rat microglia (Akundi et al., 2005). In Figure 5-9 A, PKC inhibitor staurosporine at nanomolar levels significantly reduced GABA-stimulated ROS generation in a concentration-dependent manner, supporting that PKC activity is important in GABA-induced ROS generation in activated microglia. Moreover, staurosporine significantly attenuated dieldrin-stimulated ROS generation in microglia in Figure 5-9 B. These results suggest that PKC is a mediator of ROS generation in activated microglia.

Discussion

The multi-subunit NADPH oxidase is the primary enzyme complex responsible for ROS generation in immune cells including neutrophils, macrophages and microglia (Babior, 2004). In this study, the dieldrin-induced ROS production is inhibitable by selective inhibitor DPI or apocynin, two mechanistically different inhibitors of NADPH oxidase, suggesting that the activity of NADPH oxidase is involved in the dieldrin-induced ROS production in microglia. This phenomenon is presented in both cell lines and primary microglia.

On the other hand, GABA receptors including $GABA_A$ and $GABA_B$ receptors were investigated since GABA receptors were involved in the inflammation in neutrophils. $GABA_B$ receptor may mediate cytokines IL-6 and IL-12p40 release in LPS-activated primary microglia (Kuhn et al., 2004). Both cytokine release and ROS generation are important products of microglia activation, these factors will be released into DA neurons and be detrimental to DA neurons. (Smith and Cass, 2007). With pretreatment of $GABA_A$ or $GABA_B$ antagonists, ROS generation was attenuated dramatically in a dose-dependent manner. If this action is related to antagonism on GABA receptors activity, ROS generation induced by dieldrin may be associated with GABA receptors. In hippocampal pyramidal neurons, the sensitivity of GABAergic neurotransmission to free radical such as ROS have been detected with the result that ROS effects were blocked by picrotoxin (Sah et al., 1999). Although both $GABA_A$ and $GABA_B$ receptors were involved in ROS generation in OCP-treated microglia, the complex mechanisms are unclear. It is different for a ligand to bind with ligand-gated $GABA_A$ receptor and G-protein coupled $GABA_B$ receptor, and there was no evidence that dieldrin is capable to bind with these two receptors with distinct mechanisms in microglia. The binding activities of dieldrin and two GABA receptors should be uncovered to determine the underlying mechanisms of GABA receptors involvement.

If GABA receptors activity has an effect in both cytokine release and free radicals, it is possible that their activity is related to ROS generation in activated microglia. Protein kinase C (PKC) is involved in many cellular functions including immune responses, receptor desensitization, and cell growth (Zhang et al., 2007a). It has been found that PKC activity regulate $GABA_A$ receptor in surface density and receptor sensitivity or $GABA_B$ receptor signaling efficacy (Pontier et al., 2006; Song and Messing, 2005). Moreover, PKC inhibition

leads to reduced ROS generation and cytokines release in activated microglia (Min et al., 2004). Although the precise underlying mechanisms of action remain to be delineated, our findings in brain immune cells (i.e., microglia) are in line with the reported effects of dieldrin on free radical generation in peripheral immune cells such as neutrophils (Hewett and Roth, 1988; Pelletier and Girard, 2002). Furthermore, the dieldrin-induced ROS generation in microglia was sensitive to inhibitors of gene transcription and protein synthesis. Identification of the inducible factors that contribute to the dieldrin-induced ROS production in microglia will certainly provide further insight into the related mechanisms of action. Nevertheless, increased microglial ROS production observed for dieldrin may be proven, through additional in vitro and in vivo studies, to be reminiscent of the involvement of microglial NADPH oxidase or GABA receptors in dopaminergic neurodegeneration via PKC activity.

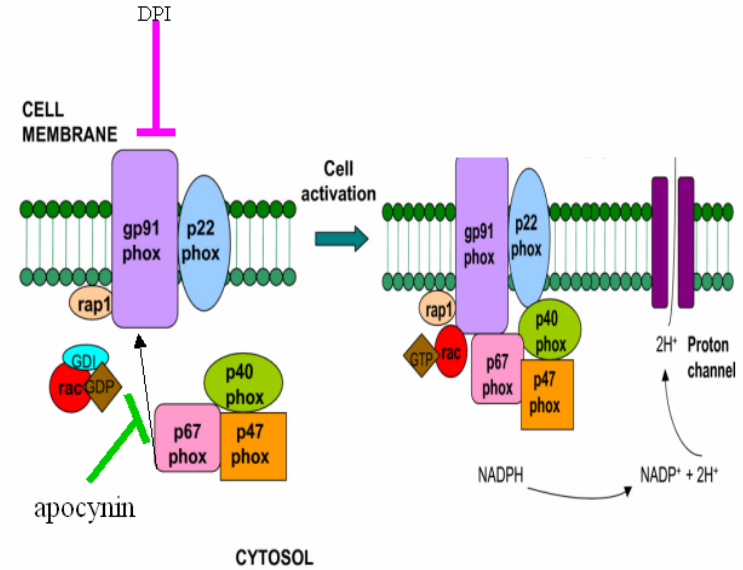


Figure 5-1. NADPH oxidase and its inhibitors DPI and Apocynin. NADPH oxidase is the primary enzyme complex for ROS gneration. NADPH oxidase has 6 subunits, two of them are cell membrane binding subunits $p22^{phox}$ and $gp91^{phox}$, and other subunits are cytosolic $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and Rac. DPI can bind with FAD to inhibit NADPH oxidase activity, and apocynin block the translocation of cytosolic part.

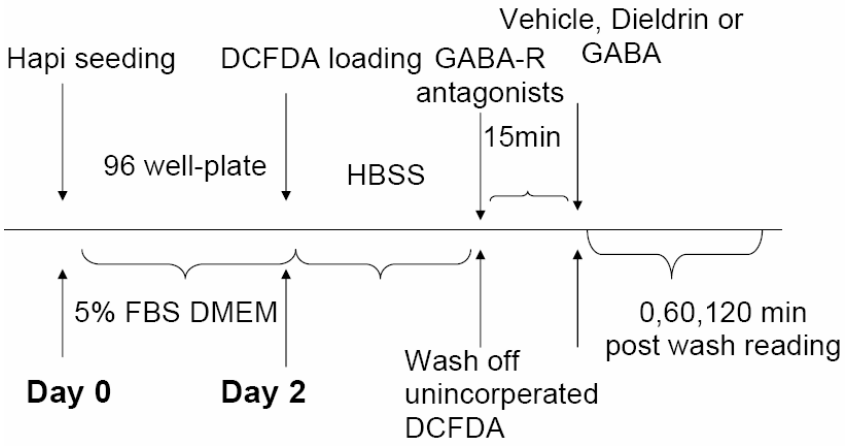


Figure 5-2. Flowchart of experimental design in aim three. DCF assay was used to detect superoxide anion generation in rat microglial cell line HAPI in 96-well plate. With 48 hours growth in serum medium, DCF was loaded for 1 hour. After DCF loading, GABA receptor antagonist picrotoxin or CGP 35348 or HBSS treated the cells for 15 min. Afterward, vehicle or dieldrin or GABA at desired concentrations treated HAPI in HBSS. The relative fluorescence unit (RFU) was read at 0, 60 and 120 min after last treatment.

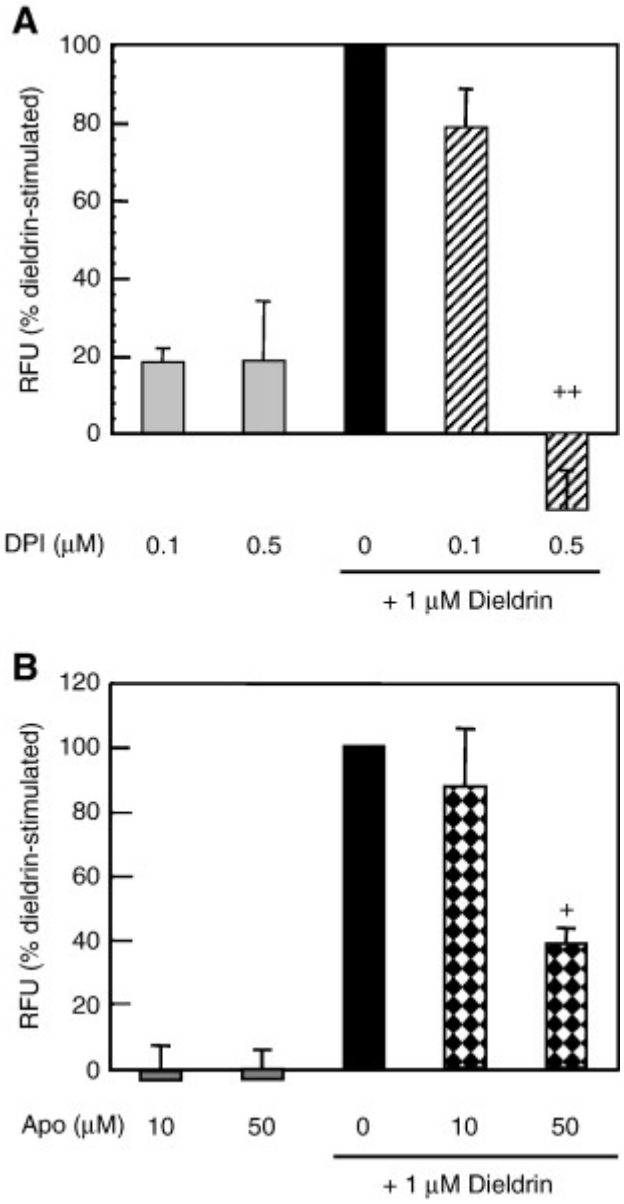


Figure 5-3. Effects of NADPH oxidase inhibitors on dieldrin-induced ROS production in rat HAPI cells. Cells were treated with medium or 1 μM dieldrin for 24 h. DPI (A) or apocynin (B) was added 18 h after the start of dieldrin treatment. Results are presented as a percentage of the dieldrin-stimulated cells and are mean ± SEM of 4–5 (A) or 3–4 (B) experiments performed in sextuplicate. $^{+}p < 0.05$ and $^{++}p < 0.005$ compared to the dieldrin-treated cells.

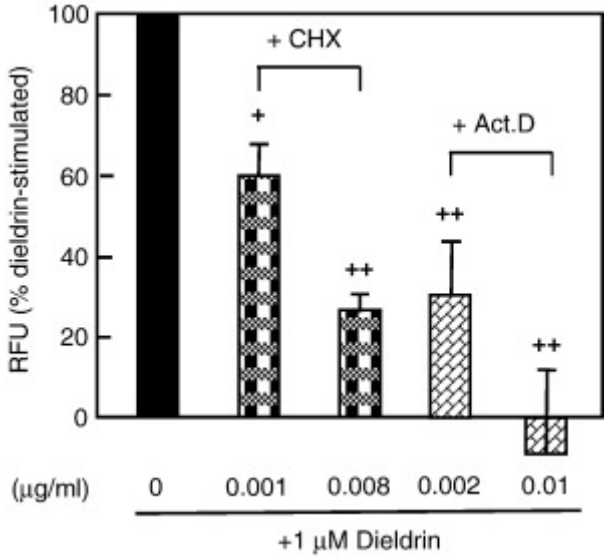


Figure 5-4. Effects of inhibitors of protein synthesis and gene transcription on dieldrin-induced ROS production in rat HAPI cells. Cells were pretreated for 15 min with medium or indicated concentrations of cycloheximide (CHX) or actinomycin D (Act. D) prior to treatment with 1 μM dieldrin for 12 h. Results are presented as a percentage of the dieldrin-stimulated cells and are mean ± SEM of 3–5 experiments performed in sextuplicate. $^{+}p < 0.05$ and $^{++}p < 0.005$ compared to the dieldrin-treated cells.

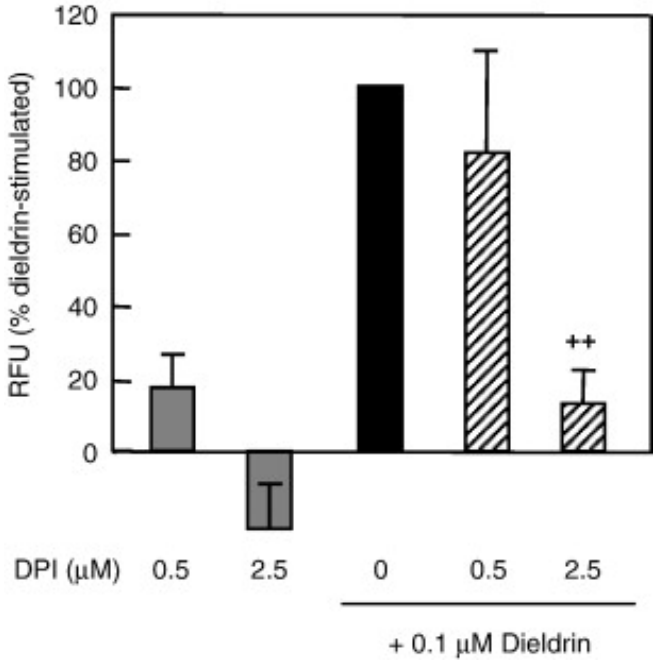
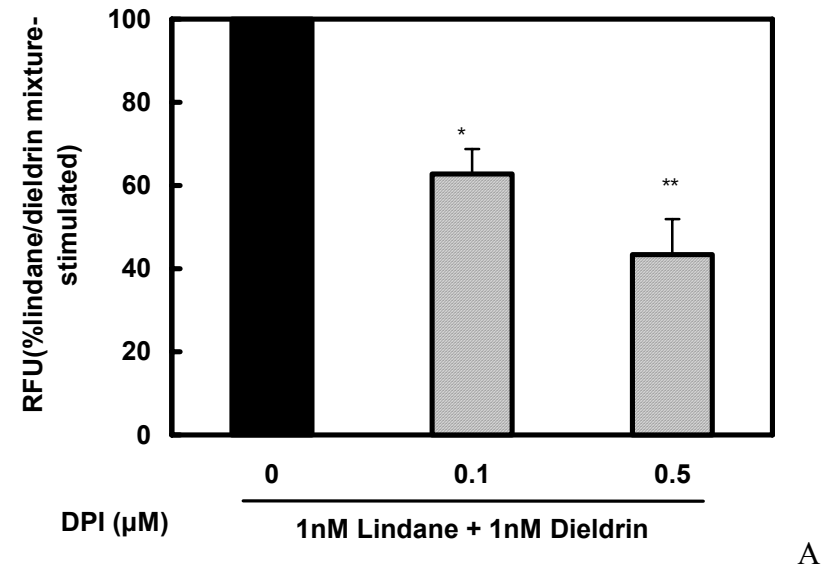
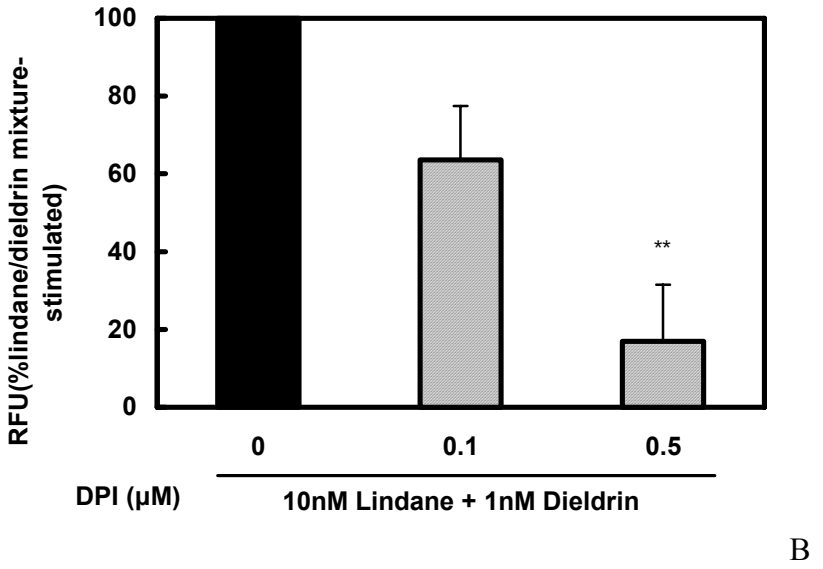


Figure 5-5. Effect of DPI on dieldrin-induced ROS production in rat primary microglia. Cells were treated with medium or 0.1 μM dieldrin for 24 h. DPI was added 18 h after the start of dieldrin treatment. Results are presented as a percentage of the dieldrin-stimulated cells and are mean ± SEM of 5 experiments performed in quadruplicate. $^{++}p < 0.005$ compared to the dieldrin-treated cells.



A



B

Figure 5-6. Effect of NADPH oxidase inhibitor DPI on lindane/dieldrin mixture-induced ROS generation in mouse BV2 microglial cells detecting by DHE assay. Results are expressed as a percentage of lindane/dieldrin mixture-induced ROS production. DPI was added 6 h before DHE loading. A. Cells were treated with medium or 1 nM lindane/1 nM dieldrin mixture for 24 h. Data are showed as mean ± SEM of three or four experiments (n=6 in each experiment). B. Cells were treated with medium or 10 nM lindane/1 nM dieldrin mixture for 24 h. Data are showed as mean ± SEM of three or four experiments (n=6 in each experiment). * $p<0.05$ and ** $p<0.005$ compared with lindane/dieldrin mixture-treated groups.

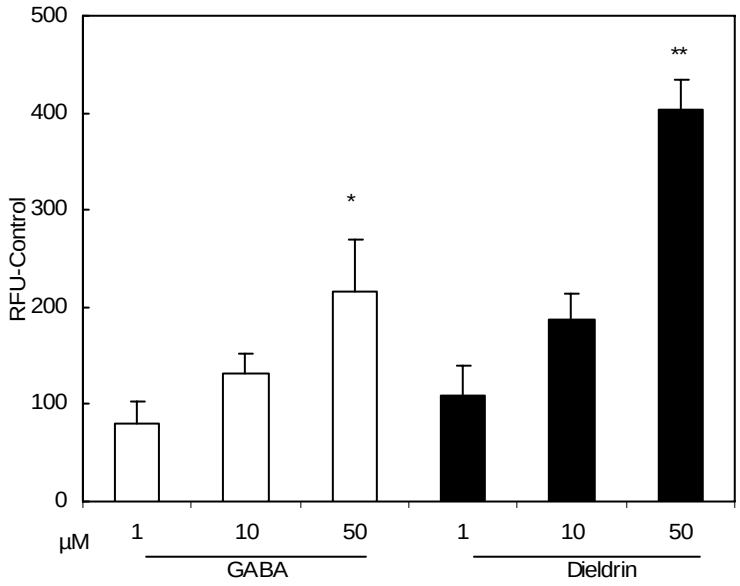


Figure 5-7. Side by side short-term effects of GABA and dieldrin-induced ROS generation in rat Hapi cells by DCF assay. Hapi cells were applied with GABA or dieldrin at 1, 10 and 50 μM for 0-2 h treatment. Generation of ROS was detected by DCF-DA (10 μM; 1h loading time). Fluorescent intensities shown to relative fluorescent units (RFU) were read at 0, 60, 120 min after GABA or dieldrin treatment. Data refer to mean ± SEM of RFU values in 60min reading with 4-6 experiments. (n=6 in each experiment). * $p<0.05$ or ** $p<0.005$ compared to time-matched corresponding lowest concentration-treated groups.

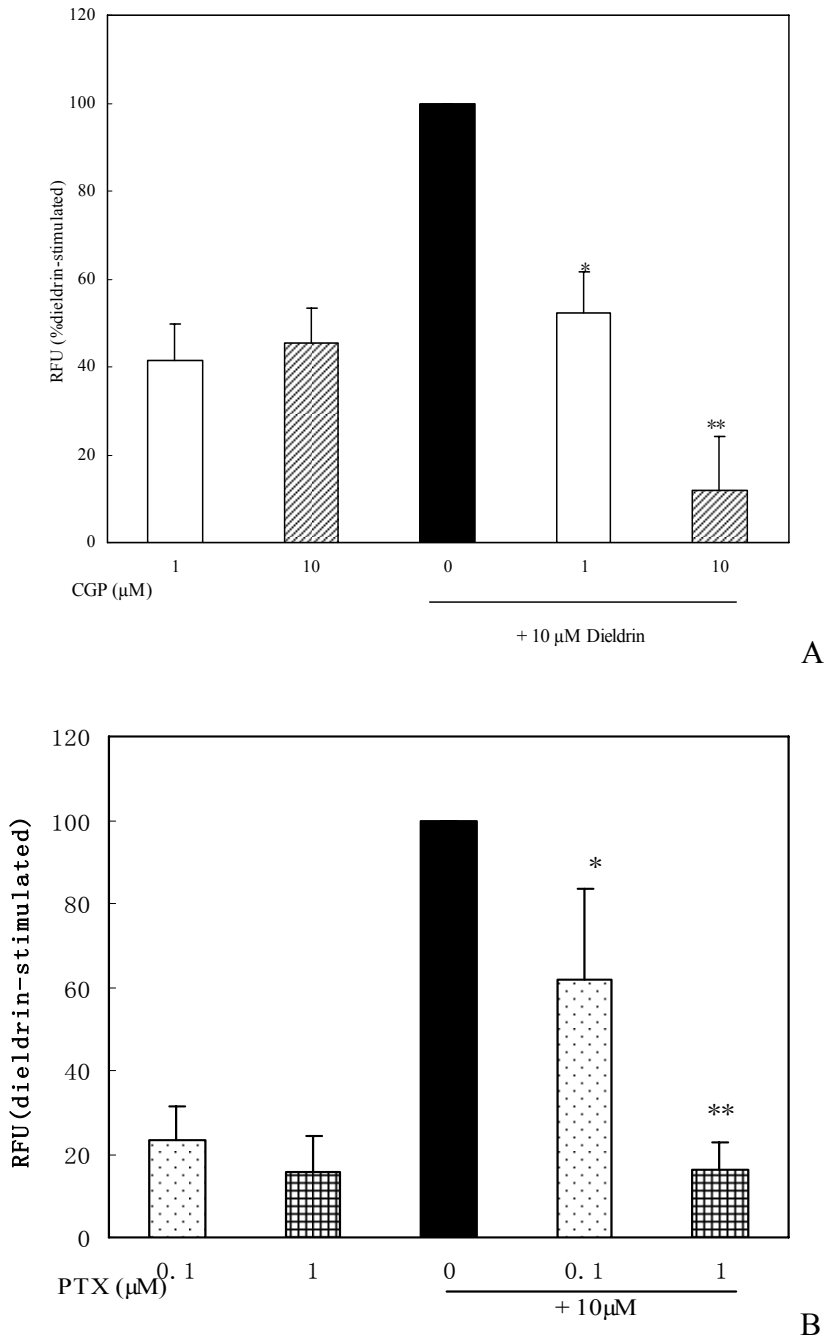


Figure 5-8. Effect of $GABA_B$ antagonist CGP 35348 (CGP) and $GABA_A$ antagonist picrotoxin (PTX) on ROS production by DCF assay. Results are expressed as a percentage of 10 μM dieldrin -induced ROS production. A. Cells were treated with medium or 10 μM dieldrin. Data are showed as mean ± SEM of three or five experiments (n=6 in each experiment). B. Cells were treated with medium or 10 μM dieldrin. Data are showed as mean ± SEM of three to six experiments (n=6 in each experiment). * $p<0.05$ and ** $p<0.005$ compared with dieldrin -treated groups.

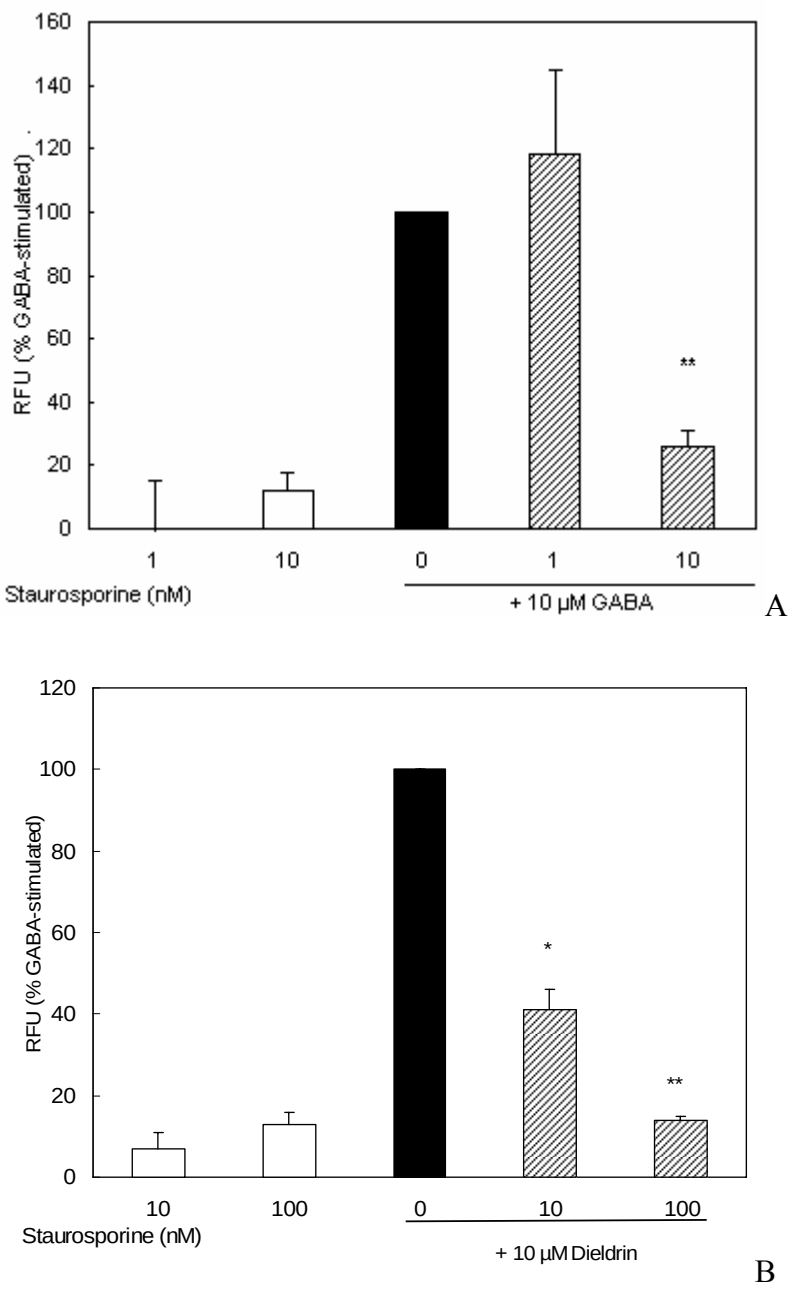


Figure 5-9. Effect of PKC inhibitor staurosporine (Stsp) on GABA or dieldrin-induced ROS generation by DCF assay. Results are expressed as a percentage of 10 µM GABA or dieldrin -induced ROS production. After 1h DCF loading, staurosporine was added 15min before GABA or dieldrin treatment. A. Cells were treated with medium or 10 µM GABA. Data are showed as mean ± SEM of three or four experiments (n=6 in each experiment). B. Cells were treated with medium or 10 µM GABA. Data are showed as mean ± SEM of three or four experiments (n=6 in each experiment). * $p<0.05$ and ** $p<0.005$ compared with dieldrin -treated groups.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The studies presented above demonstrated OCP-induced ROS generation in microglia. Being highly persistent pesticides, OCPs including dieldrin and lindane were found to effectively increase ROS generation, since microglial free radicals are key mediators of DA neurodegeneration. Environmental exposure to OCP may be a risk factor for the development of PD. Based on findings from studies of the three aims presented in previous chapters, several important conclusions can be reached. As a potential OCP related to PD development, dieldrin was hypothesized to be a microglial stimulator to induce microglial activation to release ROS. Using the DCF assay, it was observed that dieldrin induced significant increases in ROS production in a concentration -dependent manner (0.1 nM to 1 μM) and time-dependent manner (6h to 24h). Moreover, this dieldrin-induced ROS generation was consistently observed in rat HAPI microglial cells, rat primary microglia and mouse BV2 microglial cells. This dieldrin-induced ROS generation was specific to microglia but not astrocyte. Low doses treatment of dieldrin is capable of inducing significant increase in ROS from activated microglia, and the ROS may contribute to DA neurodegeneration in PD pathogenesis.

In addition to observing dieldrin-induced production of ROS in microglia, we next examined the effects of combinations of OCPs on microglial ROS generation. In the environment, multiple pesticides are present simultaneously. Lindane and dieldrin, coexist in the environments and daily food supply, and are contained in postmortem PD brains. In Aim Two, superoxide production was detected by DHE assay first in microglia treated with lindane and dieldrin alone to determine optimal concentrations of combination. Based on these results, low nanomolar concentrations were selected and four different combinations of lindane and dieldrin were then tested for their effects on microglial ROS generation. The cooperative effect of these

combinations was detected by DHE assay. Superoxide production induced by the combinations was significantly higher than that single OCP treated microglia. Synergistic effect of superoxide generation was observed for all four combinations of lindane and dieldrin at 1 or 10 nM in BV2 cells, supporting our hypothesis that chronic exposure to multiple OCPs at low doses may represent a possible environmental risk factor to the PD development. Furthermore, lindane and dieldrin at 1 nM showed the most significant synergistic effect, suggesting that exposure to low doses of lindane and dieldrin may be more relevant to PD development.

What are the underlying mechanisms of ROS generation in activated microglia induced by OCP? As a multi-subunit enzyme complex, NADPH oxidase has been associated with neurodegeneration through the formation of ROS in several PD animal models including 6-OHDA, MPTP and LPS. We hypothesized that NADPH oxidase or GABA receptors may mediate ROS generation in microglia. In Aim Three, NADPH oxidase specific inhibitors DPI or apocynin were found to inhibit ROS generation induced by dieldrin-treated or lindane/dieldrin combination-treated groups in rat Hapi cells, rat primary microglia and mouse BV2 cells. Furthermore, inhibitors of gene transcription and protein synthesis significantly reduced dieldrin-induced ROS production in rat Hapi cells, suggesting involvement of gene transcription and protein synthesis during ROS generation.

Besides NADPH oxidase, GABA receptors may also be involved in OCP-induced ROS generation in microglia. GABA, the typical GABA receptor agonist, and dieldrin were able to induce microglial ROS induction. Moreover, both $GABA_A$ selective antagonist picrotoxin and $GABA_B$ selective antagonist CGP 35348 had an inhibitory effect on ROS increase from dieldrin-treated microglia. These two antagonists significantly attenuated ROS production in a concentration-dependent manner. Finally, PKC inhibitor staurosporine significantly blocked

dieldrin-induced ROS generation in Hapi cells, suggesting PKC may be a mediator in dieldrin-induced ROS generation in microglia.

Based on these conclusions, besides OCP direct effect on DA neurons during the development of neurodegeneration, OCP indirect effect on DA neurons through microglia may be another important pathway to induce DA neurodegeneration by increased ROS production. As we know, DA neurons are relatively more vulnerable to oxidative stress than other types of neurons. In summary, OCP-induced ROS generation in microglia is mediated by NADPH oxidase and GABA receptors, and the ROS will entry into oxidative stress-sensitive DA neurons to accelerate DA neurodegeneration.

In different area of the world, the incidence of PD is different (von Campenhausen et al., 2005), and lack of data from epidemiological studies in the same area with different time period makes it difficult to study the relevance between pesticide exposure and PD. On the other hand, Epidemiology study showed the incidence of PD in individuals exposed to pesticides was 70% higher than those age and sex matched and non-exposed control (Ascherio et al., 2006). The conclusions from three aims support pesticide exposure as a risk factor in sporadic PD.

This project investigated the effect of OCP-induced ROS in microglia in a cell-based model. As for the future direction of this project, studies in animal model including OCP exposure model and NADPH oxidase subunit KO model would be a better system to determine the complex underlying mechanisms of OCP-induced ROS in microglia.

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BIOGRAPHICAL SKETCH

Haoyu Mao was born in Nanchang, Jiangxi, China, on April, 27, 1979 to Mingchu Mao and Jusun He. She is the only child in the family. Haoyu obtained her undergraduate study in Jiangxi University of Traditional Chinese Medicine with Bachelor of Science in pharmacy. After that, She earned her Master of Science in the Department of Pharmacology and Toxicology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College (now known as medical school of Tsinghua University), China. Haoyu will have graduated with Doctor of Philosophy in the Department of Pharmacodynamics, College of Pharmacy, University of Florida in Augest 2008. After graduation, Haoyu is looking forward to a new phase in her career.