THE EFFECTS OF EICOSAPENTAENOIC ACID (EPA) TREATMENTS ON THE OXIDATIVE STRESS MECHANISM IN 1-METHYL-4-PHENYL-1,2,3,6,- TETRAHYDROPYRIDINE (MPTP)-INDUCED PARKINSON'S DISEASE MODELS

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Abstract

Parkinson's disease (PD) is a progressive degenerative disorder of the central nervous system (CNS) and characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the striatum. Oxidative stress may contribute to the pathogenesis of the disease. Omega (n)-3 fatty acid, eicosapentaenoic acid (EPA), as antioxidant, may have the potential effect to prevent PD via modulating the oxidative stress.

To explore the oxidative stress mechanisms and the changes of fatty acids in PD and the modulatory function of EPA, three 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD models, cellular model, mouse brain slice model and chronic model were created. The general hypothesis of this thesis was that MPTP may increase oxidant production and destroy the balance between the oxidative and anti-oxidative systems, which may contribute to the reduction of mitochondrial activity in the cellular model of PD and contribute to the changes in the brain fatty acid profile in the animal models.

Mitochondrial dehydrogenase activity was tested by MTT assay. Oxidants were measured by 2',7'-dichlorofluo-rescindiacetate (DCF-DA) and 4,5-Diaminofluorescein diacetate (DAF-DA). Quantitative PCR was applied to measure antioxidants and pro-oxidant enzyme gene expressions. Protein expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) was measured by Western blot. Antioxidant glutathione (GSH) level was measured by a GSH assay kit. Fatty acid contents in the mouse brain were investigated by gas chromatography (GC).

In the cellular model, MPP^+ decreased mitochondrial dehydrogenase activity via over-produced oxidants, which may have been triggered by increased NADPH-oxidase. It was found that in response to oxidative activation, the concentration of the antioxidant GSH and mRNA expressions of antioxidant enzymes were increased. In the brain slice model of PD, MPTP/MPP^+ increased the n-6 fatty acids linoleic acid (LA) and arachidonic acid (AA) in the midbrain and frontal cortex. In the mouse midbrain, chronic MPTP/MPP^+ significantly decreased mRNA expressions of the antioxidant enzyme catalase and the pro-oxidant enzyme NADPH-oxidase $p47^{phox}$.

EPA up-regulated mitochondrial dehydrogenase activity in the cellular model, but it had no significant effect on the oxidative and anti-oxidative systems in any of the three models. In the mouse brain slice and chronic model, EPA treatment alone significantly increased concentrations of n-3 fatty acids EPA and docosapentaenoic acid (DPA).

In combination with MPP^+ insults, EPA reversed the MPTP/MPP^+-induced reduction of mitochondrial dehydrogenase activity and down-regulated MPTP/MPP^+-induced increases in ROS and RNS productions. EPA also attenuated the effect of MPTP/MPP^+ on mRNA and protein expressions of NADPH-oxidase and on the mRNA expression of antioxidant enzyme GSHpx.

In the brain slice study, EPA decreased the MPTP/MPP^+-induced increase in concentrations of n-6 fatty acids (LA and AA), and significantly increased concentrations of n-3 fatty acids (EPA and DPA) in the MPTP/MPP^+ treated brain slices of the PD model. In the chronic

MPTP/MPP^+-induced PD model, EPA significantly increased concentrations of n-3 fatty acids (EPA and DPA).

Together, results presented in this thesis further revealed oxidative mechanisms by which MPTP induced cell dysfunction, showed the relationship between the oxidative and anti-oxidative systems, and demonstrated the modulatory function of EPA in PD, which provide evidence for developing new treatments and for future studies.

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AAAD	aromatic L-amino acid decarboxylase
AA	arachidonic acid
Aβ	amyloid beta peptide
ACSF	artificial cerebral spinal fluid
AD	Alzheimer's disease
α-LA	alpha-linolenic acid
ATP	adenosine triphosphate
BBB	blood-brain barrier
Ca^{2+}	calcium
CNS	central nervous system
COMT	catechol-O-methyl transferase
COX-2	cyclooxygenase-2
cNOS	constitutive nitric oxide synthase
cPLA2	cytosolic phospholipase a2
DA	dopamine
DAF-2DA	4, 5-diaminofluorescein diacetate
DAF-FM	4-amino-5-methylamino-2'7'-difluorofluorescein
DAT	dopamine transporter
DCF-DA	2', 7' dichlorofluorescein diacetate
dd H2O	deuterium depleted water
DEPC	diethylpyrocarbonate

DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DMSO	dimethyl sulfoxide
DOPAC	dihydroxyphenylacetic acid
EFA	essential fatty acids
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
ETC	electron transport chain
FAME	fatty acid methyl ester
FBS	fetal bovine serum
HNE	4-hydroxynonenal
H_2O_2	hydrogen peroxide
HVA	homovanilic acid
GABA	gamma aminobutyric acid
GC	gas chromatography
GPe	external pallidal segment
GPi	internal pallidal segment
GSH	glutathione
GSHpx	glutathione peroxidase
GLA	gamma-linolenic acid
iNOS	inducible nitric oxide synthase
IL	interleukin
LA	linoleic acid

L-DOPA	3, 4-dihydroxy-L-phenylalanine
LT	leukotriene
MAO	monoamine oxidase
MDA	malondialdehyde
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine, desmethylprodine
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
N	omega
NA	noradrenaline
NADPH-oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NM	neuromelanin
NMDA	N-methylSystematic D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2^-	superoxide anion
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite radical
PBS	phosphate buffered saline
PC	protein carbonyls
PD	Parkinson's disease
PGE3	prostaglandin E3

PKC	protein kinase C
PPAR	proliferator-activated receptor
PVDF	polyvinylidene fluoride
PUFA	polyunsaturated fatty acids
RA	retinoic acid
RIPA Radio	radio-immunoprecipitation assay
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
SREBP	sterol-regulatory element binding protein
SN	substantia nigra
SNpc	substantial nigra pars compacta
SNpr	substantia nigra with its pars reticulata
STN	subthalamic nucleus
TBST	tris-buffered saline tween-20
TH	tyrosine hydroxylase
TPA	12-O-tetradecanoyl-13-phorbol acetate
VMAT	vesicular monoamine transporter
3MT	3-methoxytyramine
6-OHDA	6-hydroxydopamine
8-OHdG	8-hydroxy-2'-deoxyguanosine
8-OHG	8-hydroxyguanosine

Chapter 1

General Introduction

Summary

Parkinson's disease (PD), a progressive degenerative disorder of the central nervous system (CNS), is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the striatum (reviewed by Antoniades and Barker, 2008). Although the cause of dopaminergic neuron loss in PD is unknown, studies from post-mortem brain tissues and evidence based on animal models have strongly suggested that oxidative stress may contribute to the pathogenesis of the disease by inducing mitochondrial dysfunction and causing apoptosis (Hald and Lotharius, 2005). However, to date, the relationship between the oxidative and anti-oxidative systems in PD remains unclear. In addition, PD severely affects the quality of patients' lives, and current therapies, such as 3,4-dihydroxy-L-phenylalanine (L-DOPA), only relieve symptoms associated with early stages of the disease. In long-term PD treatments, L-DOPA may also cause adverse side effects, including inducing the oxidative stress in the late stages of the disease (Friedman and Factor, 2000; Olanow *et al.*, 2004).

Recent evidence has suggested that n-3 fatty acids, cell membrane components, including the antioxidant eicosapentaenoic acid (EPA), may have the potential to preventatively reduce the risk of PD development at both the acute and chronic stages (Bousquet *et al.*, 2008).

Currently, the most popular *in vivo* and *in vitro* models for PD research and for new treatment testing are induced by administering 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) or its metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (reviewed by Przedborski *et al.*, 2001).

As MPTP is a neurotoxin which can trigger PD-like symptoms (Burns *et al.*, 1984), the aims of the present study were to 1) characterize possible oxidative stress mechanisms of the neurotoxin-induced PD models; 2) study the effect of EPA treatment on MPTP-induced damage and 3) analyze the changes of the fatty acid profile related to the neurotoxic effect of MPTP and EPA treatments in cellular, acute and chronic models of PD.

1. 1 Parkinson's disease

PD is one of the most common progressive neurodegenerative diseases. PD symptoms include tremor at rest, rigidity, akinesia (or bradykinesia), and postural instability, which are the earliest and most striking physical symptoms, referred to as "parkinsonism" (Jankovic, 2008). PD patients also show cognitive impairments and mood disorders at the late stages of the disease (Vendrova *et al.*, 2003). These symptoms severely affect PD patients' lives and life expectancies. In Canada, PD affects approximately 100,000 people at an estimated annual cost of $5 billion (reported by CIHR 2001). Therefore, an understanding of the disease mechanism and the development of new treatments are extremely important.

1.2 Neuropathology of PD

Parkinsonism is believed to result mainly from abnormalities in the function of the basal ganglia (Marsden, 1984). Structurally, the basal ganglia consists of the striatum (caudate nucleus and putamen), the pallidum (the external and internal pallidal segments (GPe, GPi)), the subthalamic nucleus (STN) and the substantia nigra with its pars reticulata (SNpr) and pars compacta (SNpc). The basal ganglia pathways are arranged in parallel, functionally segregated circuits, divided into "motor", "associative" and "limbic" loops (reviewed by Alexander and Crutcher, 1990), all of which connect the basal ganglia with the frontal cortex. The motor loop of the basal ganglia is strongly related to the development of parkinsonism (Onla-or and Winstein, 2001), which will be discussed in detail below. The

function of the basal ganglia relies on proper dopamine (DA) functioning (reviewed by Smith and Villalba, 2008), and thus, the structure and function of the DA system will be introduced first.

DA is biosynthesized in the cell body of dopaminergic neurons. The precursor of DA, amino acid L-tyrosine, undergoes hydroxylation to become 3, 4-dihydroxy L-phenylalanine (L-DOPA) via the action of the enzyme tyrosine hydroxylase (TH). L-DOPA becomes DA through decarboxylation, via the action of the enzyme aromatic L-amino acid decarboxylase (AAAD) (reviewed by Winlow and Markstein, 1986). Following synthesis in the neurons, DA is packaged into vesicles which are stored near the pre-synaptic membrane. Upon release, DA can bind to its post-synaptic receptors, or can be degraded by auto-oxidation, which occurs in the presence of oxygen, and forms peroxides and hydroperoxides. DA can also be metabolized by catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO) in the glial cells, and be further metabolized to 3-methoxytyramine (3MT), dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA). Later, DA can be recycled by the pre-synaptic neurons via the action of the DA transporter (DAT) and then repackaged in vesicles for re-use.

In the central nervous system (CNS), DA plays a key role in motor activity, motivation, reward, sleep, mood, attention and learning (see review by Di Chiara *et al.*, 2002). DA is transmitted by four pathways, including the mesolimbic pathway, mesocortical pathway, nigrostriatal pathway and tuberoinfundibular pathway. The mesolimbic pathway (from the ventral tegmentum of the midbrain to the limbic system) plays a key role in memory and

motivation (reviewed by Wise, 2002). The mesocortical pathway (from the ventral tegmentum of the midbrain to the cerebral cortex) is involved in motivation and emotional response. The nigrostriatal pathway (from the substantia nigra (SN) to the striatum) is particularly involved in the control of movement, as a part of a system called the basal ganglia motor loop. The tuberoinfundibular pathway (from the mediobasal hypothalamus to the median eminence) regulates the secretion of hormones such as prolactin from the anterior pituitary gland (reviewed by Lieberman, 2004).

DA transmitted from the nigrostriatal pathway mainly participates in the modulation of the basal ganglia system (Smith and Villalba, 2008). The striatum is considered to be the primary input of the basal ganglia circuit. The major striatal inputs are excitory glutamatergic afferents from the frontal cortex and thalamus, which play a key role in the regulation of striatal activity (Pennartz *et al.*, 1994; reviewed by Swanson, 1996). The striatum also receives serotonergic afferents from the dosal nucleus of the raphe and caudal linear nucleus, with a small amount of noradrenergic innervations from the locus coeruleus (reviewed by Swanson, 1996). The efferent neurons of the striatum are gamma aminobutyric acid (GABA)-ergic (inhibitory), which connect with the GPe and GPi by two different pathways; the "direct pathway" and the "indirect pathway" (reviewed by Bolam *et al.*, 2000). These two pathways originate from two different subsets of the striatal neurons, which connect to the main output of the basal ganglia (GPi and SNpr) and the striatum; both require DA to function. In the direct pathways, striatal GABAergic neurons, containing dynorphin, substance P, and expressing D1 receptors, project from the GPi and SNpr directly into the thalamus. In the indirect pathway, striatal GABAergic neurons,

containing enkephalin and expressing D2 receptors, connect to the GPi and SNpr indirectly via GPe-STN-GPi projection. From the output nuclei, GABAergic neurons project into the thalamus. Thalamus nuclei, through the glutamate projection, connect to the frontal cortex. These two pathways have opposing modulatory effects in the basal ganglia loop. The direct pathway produces a disinhibition of the target area in the brain, while the indirect pathway produces an inhibition of the target area (reviewed by Swanson, 1996). DA plays an important role as a neuromodulator for the normal motor activity in the basal ganglia, and also modulates the morphological changes in striatal projection neurons (reviewed by Smith and Villalba, 2008). Therefore, high stratial DA levels contribute to a disinhibition of the basal ganglia target area, whereas low striatal DA levels produce an inhibition of the basal ganglia target area. Thus, DA is strongly related to basal ganglia functions in the brain.

In PD, the nigrostriatal dopaminergic pathway is the most damaged area in the neuropathology of the disease (reviewed by Corti *et al.*, 2005). The reason for the selective degeneration and death of dopaminergic neurons in PD remains unclear. Dopaminergic neurons in the SNpc are progressively degenerated, leading to the depletion of striatal DA concentrations, which is a neuropathological hallmark of PD (Hughes *et al.*, 1993). There is currently the suggestion that the process of neurodegeneration may start from the terminals of the dopaminergic neurons, and is possibly caused by energy impairment (due to mitochondrial dysfunction) at the neuron terminal sites (Sauer and Oertel, 1994; Sanghera *et al.*, 1997). Within this framework, neurodegeneration develops in a retrograde manner from the neuron terminals to neuron bodies in the SN through an apoptotic process, which

is one of programmed cell death (Macaya and Burke, 1992; Macaya, 1994). The loss of striatal DA can trigger prominent secondary morphological changes and affect the corticostriatal transmissions in the basal ganglia (Ingham *et al.*, 1989; Villalba *et al.*, 2009). Due to the abnormalities of basal ganglia functions in PD, the usual facilitating effects of thalamic projections to the frontal cortex are reduced. During movement, deactivation of the frontal cortex can cause a reduced motor output at the origin of motor disturbances, which induces the motor disabilities (Alexander and Crutcher, 1990; reviewed by Wichmann and DeLong, 1996).

Although PD is characterized by the loss of dopaminergic neurons, due to the involvement of the basal ganglia loop, abnormalities of GABAergic, glutamatergic, serotonergic and noradrenergic systems also occur in PD patients. The damage to the frontal cortex is related to the motor, cognitive and emotional impairment observed in PD patients (Mann and Yates, 1983; German *et al.*, 1992; reviewed by Brotchie, 2005; Schapira *et al.*, 2006). In addition, the lesion of serotonergic and noradrenergic neurons may contribute to the depression and sleep disorder symptoms seen in PD patients (Mayeux, 1984; Kostić *et al.*, 1987; reviewed by Wolters, 2006). However, as these aspects were not the objective of this study, the focus is upon the DA pathway and its relation to oxidative stress.

In PD, Lewy bodies, the other neuropathogenic outcomes, are accompanied by dopaminergic neuron loss (Dickson *et al.*, 2008). Alpha-synuclein, which was originally identified as the precursor of the non-amyloid beta peptide (Aβ) component of Alzheimer's disease (AD) amyloid (Iwai *et al.*, 1995), is a major component of Lewy bodies in PD.

Alpha-synuclein is a presynaptic terminal protein, which indicates that it may be related to dopaminergic neuron death in the neuron terminals (Baba *et al.*, 1998). It is unclear how the gene mutations affect the normal function of α-synuclein, but a possible hypothesis is that the normal localization of the protein is changed, causing α-synuclein accumulation and aggregation as a component of Lewy bodies (Iwatsubo, 2003). However, it remains unclear as to whether Lewy body formation is directly related to PD neuron degeneration. The accumulation of Lewy bodies has also been found in some brain regions in other neurodegenerative diseases (Kawahara *et al.*, 2009; Wang *et al.*, 2009). For lack of the direct evidence of the relationship between Lewy bodies and PD, this study did not focus on the Lewy body pathology.

1.3 Pathogenesis of PD

While many studies suggest that a combination of genetic, ageing and environmental factors is involved in the onset of PD, the etiology of the disease is still unclear (Moghal *et al.*, 1994; reviewed by Coppedè *et al.*, 2006). Genetic factors may be the main cause of PD. Family members of PD patients are at a 3- to 4-fold increased risk of developing the disease compared to subjects in the general population (reviewed by Coppedè *et al.*, 2006). However, the pathway of the genetic factors leading to PD is not well known. In familial PD, α-synuclein, parkin and ubiquitin carboxy-terminal hydroxylase L1 have been reported as the gene mutations that cause early onset PD. In late onset PD (idiopathic PD), there has been no gene identified to date (reviewed by Payami *et al.*, 2002).

Ageing is another factor which increases the risk of developing PD. For instance, it has been reported that the population of PD patients is mostly over 50 years old, and that the prevalence of PD in people older than 65 is much greater than that in younger people (Moghal *et al.*, 1994), implying that PD might be an age-associated process. In addition, ageing in the CNS has been associated with defects in mitochondrial respiration, increased oxidative damage and immune disorders, all of which are involved in PD pathogenesis (reviewed by Fukui and Moraes, 2008).

It has also been proposed that PD can be induced by environmental contaminants. This factor may remain unnoticed for up to several decades, but silently inflicts damage in vulnerable regions, such as the SNpc (reviewed by Petersen *et al.*, 2008). There are multiple environmental factors that have been quite consistently associated with PD, including pesticides and heavy metal exposure, contaminated food, head injuries and viral infections (reviewed by Brown *et al.*, 2005). These environmental factors may damage mitochondrial function, inducing oxidative stress and leading to dopaminergic neuron death (Corti *et al.*, 2005).

The oxidative stress mechanism in PD, which is involved in both the ageing and environmental pathogeneses, is the pathology of most interest with regards to this study. Before discussing the oxidative stress mechanism of PD, a general introduction of oxidative stress will be provided.

1.4 Oxidative stress in PD

1.4.1 Oxidants and Oxidative stress

Oxidative stress is considered to be one of the major triggers of inflammation, ageing, cancer and neurodegenerative disorders (Tsubota, 2007; Trushina and McMurray, 2007; reviewed by Mena *et al.*, 2008; Khansari *et al.*, 2009). The over-production of oxidants, or oxidative stress, may result from an imbalance between the production of oxidants and antioxidants, such as from the body's inability to readily detoxify reactive species or repair the resulting damage (reviewed by Mena *et al.*, 2008).

A particularly destructive aspect of oxidative stress is the production of reactive oxygen species (ROS), which include free radicals and peroxides. ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), the latter being a product created by the reaction between H_2O_2 and O_2^-. In general, ROS generation is the result of a cascade of reactions which starts with the production of superoxide. O_2^- can dismutate to H_2O_2 either spontaneously, or from catalyzation by superoxide dismutase (SOD). Later, the iron-catalyzed Fenton reaction can lead to the generation of OH^-, as shown in Figure 1.1. Reactive nitrogen species (RNS) are also involved in oxidative stress. The main RNS are nitric oxide (NO) and peroxynitrite radical ($ONOO^-$), as shown in Figure 1.1. The latter can be generated from a reaction between NO and O_2^-, and is highly toxic with the capacity to induce apoptosis (reviewed by Özben, 1998).

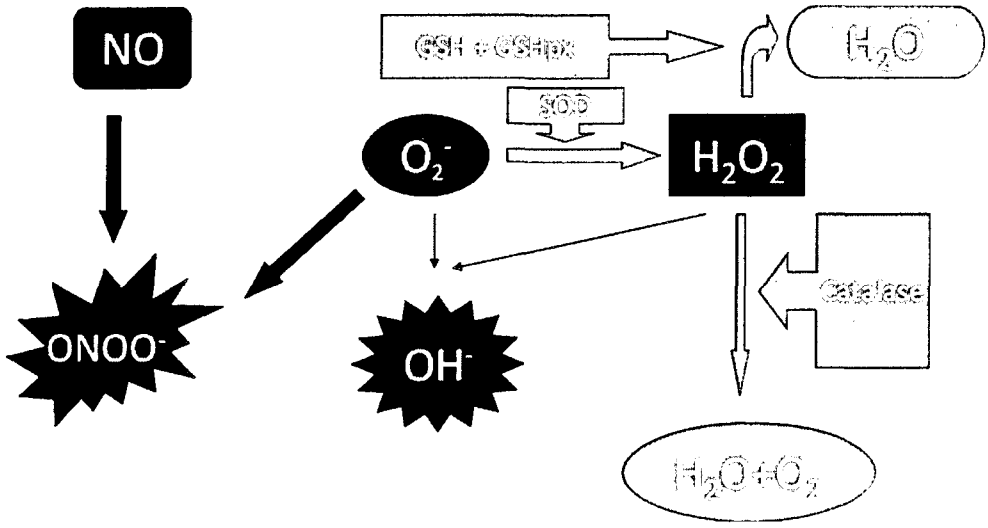

Figure 1.1. Interrelation of oxidants, antioxidants and antioxidant enzymes: O_2^-, OH^-, H_2O_2, NO, $ONOO^-$ are oxidants (toxic), which are presented against a black background. The antioxidant GSH, and the antioxidant enzymes SOD, GSHpx and catalase, as well as H_2O and O_2 (non-toxic) are presented against a grey background (Özben and Division, 1998). Figure drawn by Qingjia Meng

ROS and RNS are double-edged swords. On the beneficial side, they are released by the immune system as a weapon to attack and kill pathogens and also act as messengers between cells (Klein *et al.*, 2006; Fialkow *et al.*, 2007). ROS in microglia participate in host defense and remove debris from the CNS. RNS are also biological messenger molecules with various physiological roles. For instance, NO can modulate mitochondrial membrane potential, and influence various facets of the immune response. On the other hand, as mentioned above, the excessive and long-lasting production of oxidants can be toxic (reviewed by Linnane *et al.*, 2007). The over-production of oxidants can damage mitochondrial function and destroy the structure of proteins, lipids, and DNA, and eventually cause neuronal apoptosis and induce neurodegeneration (Li *et al.*, 2006; Ma *et al.*, 2008; Mirzaei and Regnier, 2008). Apoptosis is considered to be the main type of programmed cell death in PD, which involves a series of biochemical events leading to characteristic cell morphology and death. Characteristic morphology of cells undergoing apoptosis includes blebbing, cell membrane changes, such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. In contrast with the other cell death mechanism, necrosis, which results from acute cellular injury, apoptosis occurs as a defense mechanism such as in immune reaction or when cells are damaged by disease or noxious agents. However, the overlap between these two processes remains, and they may together contribute to cell death. Apoptotic cells showing several biochemical modifications such as protein cleavage, protein cross-linking and DNA breakdown are believed to be related to the oxidative stress mechanism in PD (Loh, 2006; Elmore, 2007). The details of the induced damage from oxidative stress in PD will be discussed as follows.

Firstly, lipids are essential components of the cell membrane. Any severe impairment of the cell membrane is an irreversible process, which can induce cell death. Lipid peroxidation, induced by the over-production of oxidants, is one of the pathogenic outcomes of cell membrane impairment. $OH^{\cdot}$ is extremely harmful to the cell membrane as well. It can extract one hydrogen atom from the methylene groups of fatty acids (essential lipid components). After this reaction, a lipid radical is generated. The recombination of two radicals can produce a stabilized radical, called lipid hydroperoxide (LOOH), which cannot be removed by antioxidants and can cause neuronal injury (reviewed by Benzie, 1996). The biomarkers of lipid peroxidation are malondialdehyde (MDA), 4-hydroxynonenal (HNE) and isoprostane (reviewed by Mosley *et al.*, 2006).

Secondly, oxidants cause protein oxidation by oxidizing free or protein-bound amino acids. The oxidation of proteins can alter protein conformation and lead to either a gain or loss of protein function, resulting in the deactivation of enzymes, caused by interruption of membrane channels and pumps and the active sites of enzymes, a process which precedes apoptosis (reviewed by Schopfer *et al.*, 2003; reviewed by Hasan *et al.*, 2007). The protein peroxidation biomarker, protein carbonyl (PC), is formed following protein modification by oxidative damage (Mosley *et al.*, 2006).

Thirdly, nucleic acid damage is caused by an over-production of oxidants. This damage can result in the disruption of transcription, translation and DNA replication. Mutations in nuclear or mitochondrial DNA can disturb cellular metabolism, leading to cell apoptosis.

As well, ageing may reduce the efficiency of DNA repair in organisms, which may explain the relationship between PD and age (reviewed Rao and Loeb, 1992). The biomarkers of oxidative damage to nucleic acids are nucleoside 8-hydroxyguanosine (8-OHG) for RNA and/or 8-hydroxy-2'-deoxyguanosine (8-OHdG) for DNA (Battisti *et al.*, 2004).

Together, the damage of lipids, proteins and nucleic acids all contribute to the impairment of cell function, such as mitochondrial dysfunction, which contributes to the neuronal energy deficiency observed in PD and neuron apoptosis (Niizuma *et al.*, 2009). However, the damages discussed above are only induced when enzyme or non-enzyme antioxidants are insufficient to control oxidants.

1.4.2 Antioxidants and antioxidant enzymes

Antioxidants play important neuroprotective roles. Antioxidant defense exists in nearly all types of cells exposed to oxygen. Antioxidants and antioxidant defenses are mediated by antioxidant enzymes, including SOD, glutathione peroxidase (GSHpx) and catalase (Figure 1.1). SOD is homogeneously distributed in all the brain regions (Thomas, 1976) and can remove O_2^- by catalyzing the dismutation of O_2^- into H_2O_2. Failure of SOD activity can increase the production of O_2^- in the electron transport chain (ETC) and aggravate inflammatory processes, because catalase and GSHpx are deactivated by O_2^-. Following conversion of O_2^- to H_2O_2, the removal of H_2O_2 and the repair of oxidized protein thiols are either catalyzed by GSHpx (GSH's co-enzyme) by coupling the reduction with the oxidation of glutathione (GSH) or via the action of another antioxidant enzyme, catalase. A study of the distribution of catalase found that the highest activity of catalase in the brain is

in the hypothalamus and SN, and the lowest activity is in the striatum and frontal cortex (Brannan *et al.*, 1981). Catalase can catalyze the decomposition of H_2O_2 to H_2O and O_2 (reviewed by Özben, 1998).

However, Oshino and Chance (1977) elucidated that GSHpx may be more important than catalase in the brain, because catalase concentrations within the mitochondria are low. GSHpx activity is high in the SN, which indicates a greater need for peroxide-detoxifying enzymes in the dopaminergic neurons. However, GSHpx activity in glial cells is higher than in neurons. It is known that MAO, which metabolizes DA and produces oxidants, is also mainly localized in the glial cells, which may indicate that DA production is related to GSHpx content (Spina and Cohen, 1989). The antioxidant GSH is the most abundant form of small molecule, non-protein thiol, present in all animal cells. During the reaction of GSH and GSHpx, GSH serves as an electron donor, and glutathione disulfide (GSSG) is formed. GSH is an essential player in the cellular defense system (Benzi *et al.*, 1990) and can prevent membrane lipid oxidation (Thomas *et al.*, 1990). Maintaining GSH levels is very important for cellular metabolic regulation (Miller *et al.*, 1990). However, GSH production is adenosine triphosphate (ATP)-dependent, thus, mitochondrial respiration impairment can result in reduced GSH synthesis (reviewed by Meister, 1991). In addition, it has been found that there are decreased levels of GSH in the brain during ageing, indicating that increased oxidative stress may be related to cell ageing (Sohal and Allen, 1990; Benzi *et al.*, 1992).

1.4.3 Sources of oxidants

According to the producing source, oxidants can be divided into two types: non-enzyme-

dependent and enzyme-dependent.

1.4.3.1 Electron transport chain

Predominantly, the non-enzyme-dependent ROS originate from the ETC in the mitochondria, where the reaction between an electron donor (such as NADH) and an electron acceptor (such as O_2) transfers H^+ ions across a membrane through a series of biochemical reactions. ATP, the essential energy component in mammals, is a product of this process. The ETC transfers H^+ to O_2 through five associated proteins in the following order: from NADH to complex I, and then coenzyme Q, complex III , cytochrome c, and complex IV, and finally to O_2. In the mitochondria, complexes I, III and IV are proton pumps, while coenzyme Q and cytochrome c are mobile electron carriers (complexes are defined as multiple linked proteins). Four consecutive electrons are required for one oxygen molecule to be converted into H_2O. However, a small percentage of oxygen molecules (1%-2%) are converted into O_2^- as a result of a two-electron reduction from complex I and complex III (reviewed by Davidson and Sittman, 1999). It has been shown that the inhibition of complex I and complex III contributes to O_2^- production (Turrens, 1997), causing higher numbers of electrons to escape from the chain, leading to ROS formation and lower ATP levels (Mrácek *et al.*, 2006) (Figure 1.2). H_2O_2 is produced as a secondary product from the reaction between O_2^- and H_2O. Furthermore, the OH^- radical is produced by the further reduction of H_2O_2 and O_2^-.

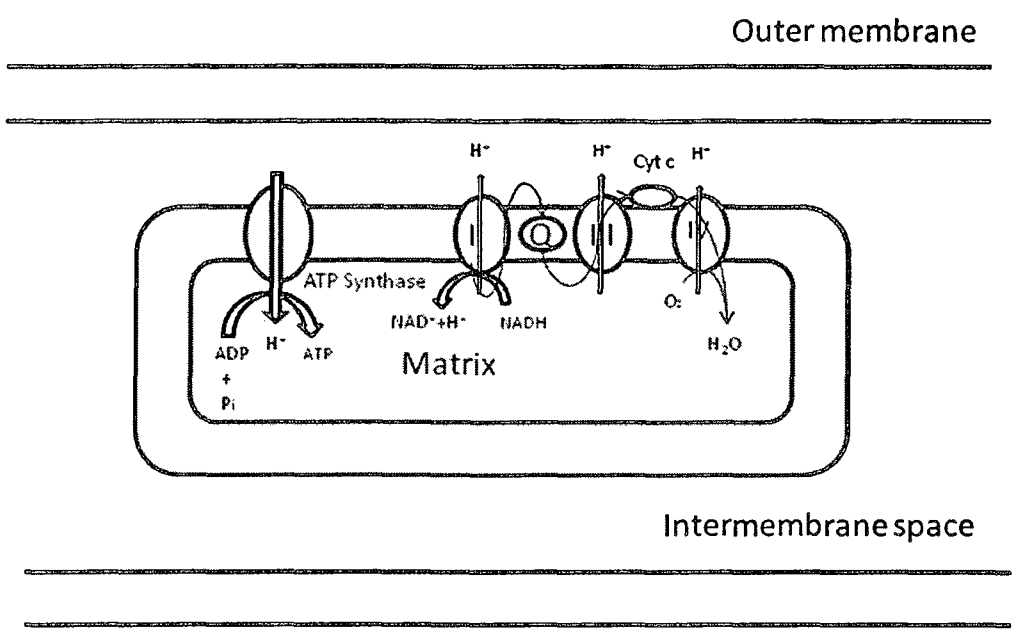

Figure 1.2. The ETC in the mitochondria: ETC transfers H^+ to O_2 though five associated proteins from NADH, to complex I, coenzyme Q, complex III, cytochrome c, and complex IV, to O_2. The NADH generated in the citric acid cycle is then oxidized, providing energy to power ATP synthase (Davidson and Sittman, 1999). Figure drawn by Qingjia Meng

Most RNS productions are enzyme-dependent, while the source of non-enzyme RNS is not well known.

1.4.3.2 Pro-oxidant enzymes

In the mitochondria, oxidants can be produced by two major enzymes, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) and nitric oxide synthase (NOS). NADPH-oxidase is involved in an inducible electron transport system that transfers reducing equivalents from NADPH to oxygen, producing cellular O_2^- (reviewed by Babior, 2004). In the CNS, microglia may be the main type of cell using this enzyme to produce O_2^- (Zhang *et al.,* 2004). A similar event may also occur in neurons (Park and Jin, 2008). NADPH-oxidase is a multimeric enzyme composed of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits. In resting microglia, NADPH-oxidase is inactive because $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ exist in the cytosol as a complex, and are separated from the transmembrane proteins $gp91^{phox}$ and $p22^{phox}$. Upon microglial activation, $p47^{phox}$ is phosphorylated and the entire cytosolic complex translocates to the membrane, where it combines with $gp91^{phox}$ and $p22^{phox}$, forming NADPH-oxidase (reviewed by Babior, 1999).

NADPH-oxidase is normally localized in two types of cells: microglia and neurons. Microglia, one type of glial cell, are non-neuronal cells that provide support and nutrition, maintain homeostasis, and participate in signal transmission in the CNS, and act as the first and main form of active immune defense (Figure 1.3). The microglial cells are also integral to normal CNS functioning, since they are essential in removing dead cells or their

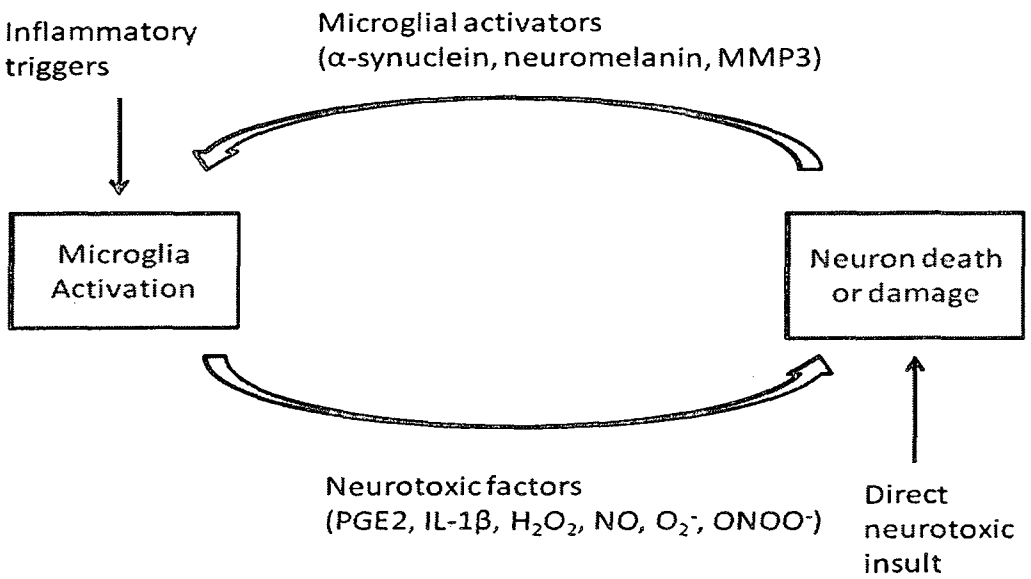

Figure 1.3. Microglial activation: A resting microglial cell can be activated by specific stimuli, such as pro-inflammatory triggers, cytokines, aggregated Aβ, oligomers, fibrillar Aβ and plaque, and neuronal death or damage. Activated microglia can produce cytokines and oxidants, which have the ability to induce neuronal damage (reviewed by Murphy 2000, drawn by Qingjia Meng).

remnants by phagocytosis, and promoting tissue repair. However, when over-activated, they can be toxic to neurons (reviewed by Male *et al.*, 2006) since they can release cytotoxic substances. These substances include ROS and proinflammatory cytokines, which can damage neuronal function, integrity and chances of survival. Microglia are activated by damaged neuronal products such as pathogenically-modified CNS proteins and cytokines which are induced by neuron damage (Sugama *et al.*, 2003). Upon microglial activation, increases in intracellular Ca^{2+} can activate protein kinase C (PKC) (Light *et al.*, 2006; Wang *et al.*, 2006). PKC can phosphorylate the serines of $p47^{phox}$ and activate NADPH-oxidase to produce O_2^- in the microglia, which can enter into the neuron (Fontayne *et al.*, 2002).

In neurons, the mechanism of NADPH-oxidase activation is still unclear. While one study showed that an overload of zinc may induce PKC activation, leading to PKC activation of neuronal NADPH-oxidase (Noh and Koh, 2000). In addition, increased levels of Ca^{2+} can also induce activation of the Ca^{2+}-dependent enzyme NADPH-oxidase in the neuron (reviewed by Shibata and Kobayashi, 2008).

The other enzyme, NOS, is the key enzyme involved in the biosynthesis of NO, and plays an important role in cellular communication (Wu *et al.*, 2003). There are three known isoforms of NOS, two of which are constitutive (cNOS), while the third is inducible (iNOS). cNOS includes both neuronal constitutive (nNOS) and endothelial constitutive (eNOS) (reviewed by Förstermann *et al.*, 1995). Among these three isoforms, nNOS and iNOS in particular have implications in PD pathogenesis. nNOS is the main isoform of NOS,

constitutively expressed in neurons and glial cells. The other isoform iNOS can be expressed in brain glial cells and active macrophages in response to a variety of neuronal damage and can be activated by cytokines, such as TNF-α, ROS in the microglia, and pathogenically modified CNS proteins (Levecque *et al.*, 2003). Following activation, NO production can be produced by iNOS (reviewed by Murphy, 2000).

1.4.4 Mechanisms of oxidative stress in PD

Mitochondrial dysfunction, DA oxidation, and microglial activation are believed to be the most important pathways involved in the production of oxidants in PD (Mosley *et al.*, 2006). These pathways do not, however, function independently.

1.4.4.1 Mitochondrial dysfunction

In 1989, the role of mitochondrial dysfunction in PD was first reported in the SNpc of PD patients (Schapira *et al.*, 1989). Thereafter, a study on MPTP-induced *in vivo* and *in vitro* models showed the normal flow of electrons in the respiratory chain to be blocked when complex I is inhibited in PD, causing more electrons than usual to escape from the chain, leading to ROS formation and lower ATP levels (reviewed by Przedborski and Vila, 2003). Oxidants from the ETC chain can further induce the activation of excitatory amino acid receptors, resulting in an increased intracellular Ca^{2+} concentration. Higher Ca^{2+} activates the Ca^{2+}-dependent enzyme NADPH-oxidase (reviewed by Shibata and Kobayashi 2008) and nNOS (reviewed by Murphy 2000). nNOS can induce NO production, which, by reacting with O_2^-, could produce $ONOO^-$. $ONOO^-$ is extremely toxic to the cells, leading to cell damage and triggering apoptosis (reviewed by Poderoso, 2009). Oxidants resulting

from mitochondrial dysfunction also induce the collapse of mitochondrial membrane potential (reviewed by Henchcliffe and Beal, 2008), due to its initiation of an extremely detrimental positive feedback signaling loop (reviewed by Mizuno *et al.*, 2008).

1.4.4.2 DA oxidation

An analysis of the postmortem tissue of PD patients showed that although extensive loss of DA neurons occurred, only minor clinical symptoms appeared (Bernheimer *et al.*, 1973; reviewed by Agid, 1991). The clinical symptoms observed mainly resulted from DA depletion. However, the number of lost DA neurons observed in the absence of any association with clinical symptoms, indicates that compensatory DA mechanisms may exist. Such typical compensatory mechanisms include the increased release of DA from remaining dopaminergic neurons. Indeed, the phenomenon of increased DA release and metabolism has been reported in the brains of PD patients (Bezard and Gross, 1998), as indicated by substrate ratio (i.e. DOPAC/DA). A lower concentration of the DA transporter, DAT, is another compensatory mechanism to maintain a sufficient DA concentration within the striatum, which contributes to the maintenance of normal motor behavior, during the preclinical phase of PD (Cruz-Muros *et al.*, 2007). Although the compensatory system can maintain an adequate DA concentration, it can also trigger oxidative stress (Loeffler *et al.*, 1994). Eventually, in the late stage of PD, when the majority of dopaminergic neurons are lost, the compensatory system would be unable to maintain appropriate DA concentrations. The compensatory mechanism explains why PD symptoms emerge only after striatal DA becomes depleted by 70% or more (reviewed by Hornykiewicz, 1975). There are two mechanisms by which DA can be oxidized, both of which have been demonstrated in the

MPTP *in vivo* model (Chiueh *et al.*, 1994). The first one occurs when enzyme MAOs break down DA to its metabolites. Usually DA is very stable in synaptic vesicles, but if excessive amounts of cytosolic DA exist outside of the synaptic vesicles, then DA is readily metabolized by MAO, leading to the production of DOPAC and H_2O_2 by the reaction between O_2^- and H_2O (Maker *et al.* 1981; reviewed by Gesi *et al.*, 2001; Miyazaki and Asanuma, 2008). In addition, MAO activity is increased during ageing, which may cause increased DA oxidation (Alper *et al.*, 1999). Thus, following this theory, the DA complementary system mentioned above may produce even more oxidants. From this reaction alone, dopaminergic neurons can be exposed to oxidative stress. The second procedure is referred to as DA auto-oxidation. DA is easily oxidized in abnormal conditions such as exposure to endogenous and environmental stresses as well as pathological conditions. For example, during an inflammatory response, levels of cyclooxygenase-2 (COX-2) are increased and more DA oxidation occurs, which in turn produces H_2O_2 and DA-quinone (Madrigal *et al.*, 2003; Chae *et al.*, 2007). DA-quinone can readily bind to and modify proteins in the cell, such as TH, DAT and parkin protein. DA-quinone could consequently deactivate those molecules and lead to a disturbance in DA synthesis and metabolism (Rabinovic *et al.*, 2000). DA-quinone is also closely related to the stabilization of α-synuclein, which can later induce Lewy body formation (reviewed by Hastings and Zigmond, 1997). Of the two procedures, DA auto-oxidation is believed to be the primary source of DA oxidation (Chae *et al.*, 2007).

1.4.4.3 Microglial activation

The highest density of glial cells has been found in the SNpc of the brain (Lawson *et al.*,

1990), and activated microglia are commonly observed in the SNpc of PD brains. Microglia can be activated by signals (pathogenically modified CNS proteins and cytokines, such as TNF-α) released from degenerating neurons (Figure 1.3). As well, activated microglia extend to the damaged area and shield the area from healthy tissue (Kim and Joh, 2006). Following activation, pro-oxidant enzymes in microglia become active and produce oxidants to attack and kill pathogens to protect the neuron (Choi *et al.*, 2005). However, over-activated pro-oxidant enzymes can lead to the production of excessive oxidants, which can damage the neuron. In postmortem SNpc of patients with PD, protein expression of the NADPH-oxidase $gp91^{phox}$ located in the microglia and mRNA expression of iNOS activity were increased (Eve *et al.*, 1998; Wu *et al.*, 2003). Thus, overactivated microglia are another source of oxidant production.

In summary, environmental toxins such as pesticides, and age-related immune disorders can all cause neuronal cell ageing and damage. Neuronal damage may induce the activation of the microglia, which can then release oxidants and cause further inflammation. Inflammation is a protective mechanism in the body and brain to fight foreign invaders and infections, and remove the dead tissues and injurious stimuli. For instance, activated microglia can produce mediators, such as cytokines and oxidants, to destroy the invading infection, and phagocytose dead cells or tissues. However, chronic inflammation may cause neuron damage. Additionally, this oxidative damage may also cause mitochondrial dysfunction, inducing further oxidant production. DA compensatory mechanisms also trigger further oxidant release. These oxidants together cause DA oxidation, which may contribute to dopaminergic neurodegeneration and the induction of PD. However, it

remains unclear as to which factor is the original cause of oxidative stress. Whether oxidative stress induces neuronal apoptosis and neurodegeneration initially or whether neuropathological changes and neuron damage result in oxidant production is a circular question.

1.4.5 Evidence of oxidative stress in PD

Changes in the oxidative system are involved in the neuropathology of PD, including increased oxidant production and reduced anti-oxidative protection. Oxidative damage to proteins, lipids and DNA (PC, MDA and 8-OHdG) were found to be increased in the SNpc of autopsied PD patients (reviewed by Mosley *et al.*, 2006) as well as in MPTP/MPP^+ treated animals and cells (Chiueh *et al.*, 2000; Ortiz *et al.*, 2003). In addition, one main subunit of NADPH-oxidase, $gp91^{phox}$, was up-regulated in the SNpc of PD patients, and $gp91^{phox}$ was also found in the MPTP mouse model (Wu *et al.*, 2003). Higher expressions of the neuronal and inducible NOS enzymes were found in the nigrostriatal region and basal ganglia in both postmortem PD brains (Eve *et al.*, 1998) and the MPTP model (Himeda *et al.*, 2006). The theory that oxidative stress is related to the initiation of dopaminergic neuron loss in the substantia nigra, which can induce PD (reviewed by Hirsch, 1993; reviewed by Jenner and Olanow, 1996; reviewed by Hald *et al.*, 2007), is also supported by the MPTP-treated mice (Paterna *et al.*, 2007). At the same time, decreased levels of the major antioxidant GSH was found in the brain of PD patients (Zoccarato, 2005), which has been confirmed in MPTP-treated mice and cellular models (Zeevalk *et al.*, 2007; Thomas *et al.*, 2008). In addition, SOD and catalase activities in patients with PD were reduced (de la Torre *et al.*, 1996). Together, these pieces of evidence show that

changes in both oxidative and anti-oxidative systems are associated with PD, indicating that oxidative stress plays an important role in the pathogenesis of PD. However, different findings from certain antioxidant studies show some controversy. For instance, SOD activity was found to be increased in the SN of PD patients (Marttila *et al.*, 1988), and the interplay between oxidants and antioxidants remains unclear.

1.5 PD models

To understand the pathogenesis of PD, and to investigate new therapeutic treatment mechanisms for PD patients, it is important to have relevant disease models of PD. Human neurological disorders can be modeled in animals according to standardized procedures which have face validity (behavioral outcomes), construct validity (specific pathogenic features) and predictive validity (the ability to correctly identifying the efficacy of a putative therapy) (van der Staay, 2006). PD models currently recreate many of the main symptoms or pathological changes of PD, including (1) the development of selective lesions of dopaminergic neurons in the SN with ageing; (2) a significant reduction of striatal DA levels; (3) damage from oxidative stress, mitochondrial dysfunction and microglial activation; (4) the formation of Lewy bodies; (5) the development of striking parkinsonism, and (6) identification of efficient anti-PD drugs.

To induce PD *in vivo* and *in vitro* models, several neurotoxins, such as MPTP, 6-hydroxydopamine (6-OHDA) and rotenone are often used. According to the above

mentioned standards for PD models, the evaluation of these models will follow in the subsequent section.

1.5.1 6-Hydroxydopamine

6-OHDA was the first identified catecholaminergic neurotoxin (Sachs and Jonsson, 1975) and has been popularly applied to rats (reviewed by Eslamboli *et al.*, 2005). 6-OHDA can cause the specific degeneration of catecholaminergic neurons in many brain regions by using the same catecholamine transport and reuptaking transporter systems as DA and norepinephine. 6-OHDA produces a slow, retrograde degeneration of the nigrostriatal systems. Extensive DA loss has been found in most studies using this model. The oxidative stress mechanism is involved in 6-OHDA neurotoxicity via the depletion of mitochondrial complex I (reviewed by Simola *et al.*, 2007), but MAO-B inhibitors can prevent the neurotoxic effect of 6-OHDA (Knoll, 1986). However, the 6-OHDA model, as a model of PD involves several drawbacks: 1) 6-OHDA is unable to cross the blood-brain barrier (BBB) and requires stereotactic injection into the substantia nigra, the nigrostriatal tract or the striatum to specifically damage the nigrostriatal dopaminergic pathway (reviewed by Perese *et al.*, 1989); 2) the 6-OHDA model does not produce all the clinical and pathological features of PD. For instance, it cannot induce Lewy body formation, which is a neuropathological marker of PD and 3) this model exclusively induces acute effects, and cannot mimic the slow and progressive pathology of human PD. In addition, it can only produce rotation but not other behavioral outcomes (reviewed by Schober, 2004; Bové *et al.*, 2005).

Although 6-OHDA has limitations, it has been used as a model to test the efficacy of PD therapies for many years, and is also a good model to test oxidative stress mechanism in PD (reviewed by Schober, 2004).

1.5.2 Rotenone

Rotenone is a pesticide that is derived from the roots and stems of plants such as the jicama vine plant (Sae-Yun *et al.*, 2006). Rotenone is able to freely cross cellular membranes, and consequently accumulates in sub-cellular organelles, such as mitochondria, where it impairs oxidative phosphorylation by inhibiting complex I of the ETC (Schuler and Casida, 2001). Rotenone results in a uniform mitochondrial complex I inhibition throughout the brain. It also causes the degeneration of dopaminergic neurons and decreased DA levels (Saravanan *et al.*, 2005). However, it remains unclear as to whether complex I inhibition induced by rotenone is directly related to DA damage. Unlike the 6-OHDA model, the formation of Lewy bodies has been found in this model (Huang *et al.*, 2009). Some studies reported that rotenone can successfully produce parkinsonism, which was successfully antagonized by L-DOPA (Alam and Schmidt, 2004); whereas other studies reported that it fails to induce PD symptoms in mice or rats (Lapointe *et al.*, 2004; Rojo *et al.*, 2007). In addition, one study showed that rotenone-induced lesions were variable in different types of animals (Betarbet *et al.*, 2002). Despite these limitations, rotenone has been used to investigate Lewy body formation and complex I inhibition in PD.

1.5.3 MPTP model

MPTP is a neurotoxin which can selectively destroy dopaminergic neurons in the SNpc.

MPTP models, such as the one employed in this study, have been commonly used as dopaminergic neurotoxins because they induce a severe parkinsonian syndrome accompanied by the loss of dopaminergic cells in the SNpc of mice, non-human primates and humans (reviewed by Kopin, 1987; Heikkila and Sonsalla, 1987). These models can also exert similar effects in other animals, including dogs, cats, sheep, and even goldfish (Zigmond and Stricker, 1989; Gerlach *et al.*, 1991; Heikkila and Sonsalla, 1992; Tipton and Singer, 1993), with no severe effect in rats or guinea pigs. Biochemical and histological studies have demonstrated that the MPTP-induced parkinsonian syndrome in humans exactly matches neuropathologic outcomes seen in PD (Gerlach *et al.*, 1991). Since MPTP can cross the BBB, it can be systemically injected. Its neurotoxic effects can be reduced by L-DOPA (Domino and Sheng, 1993). However, some studies have elucidated that MPTP models are not ideal for PD research because MPTP only produces transient, short-term behavioral symptoms, coupled with non-specific symptoms such as hypersalivation, convulsions, piloerection, and hypokinesia (reviewed by Schober, 2004). In addition, although MPTP is able to replicate the loss of the dopaminergic pathway in a short time, recovery occurs after several days (O'Callaghan *et al.*, 1990a). For instance, in mice, following initial acute MPTP effects, recovery is rapid and motor deficits are unapparent (Sundström *et al.*, 1990). Even after chronic MPTP treatment, the recovery of striatal and nigral TH immunoreactivity and motor deficits were observed within 6 months. In behavioral tests and biomarker assays, individual differences in the MPTP animal model were also observed (reviewed by Gerlach and Riederer, 1996). It is possible that individual variability in the sensitivity of dopaminergic neuronal systems and the biological variability in the function of compensatory mechanisms play a key role. As well, MPTP cannot

produce Lewy bodies. Generally, MPTP treatment usually induces acute lesions, but fails to cause the progressive nature of PD, whereas prolonged treatment may overcome this limitation. Although there are limitations of the MPTP models, MPTP currently represents the most important and most frequently used parkinsonian toxin employed in animal models (reviewed by Beal, 2001; reviewed by Przedborski *et al.*, 2001) and has a competitive advantage over all other toxic PD models because: (i) it directly causes a specific intoxication of dopaminergic structures; (ii) it induces human symptoms virtually identical to PD and (iii) it results in the same pathology, oxidative stress, mitochondrial dysfunction and inflammation observed in PD (reviewed by Przedborski and Vila, 2003). The mechanisms of MPTP action will be discussed as follows.

1.5.3.1 Mechanisms of MPTP action

MPTP enters the brain by crossing the BBB. Thereafter, MPTP is taken up by a type of glial cell referred to as astrocytes, and converted to its active form, MPP^+, via actions of the enzyme MAO-B. Then, MPP^+ is released from the astrocytes into the extracellular space, and can be specifically transported into dopaminergic neurons via DAT. Inside the dopaminergic neuron, MPP^+ can be concentrated in the mitochondria, or stored in synaptic vesicles. After entering the nigral neurons, MPP^+ can be accumulated up to 40-fold in the neuronal mitochondria. This accumulation apparently results from the energy-dependent electrical gradient that is maintained across the mitochondrial membrane. Upon accumulating in the mitochondria, MPP^+ inhibits the complex I of the ETC and causes oxidant overproduction (O'Callaghan *et al.*, 1990b; Lotharius and O'Malley, 2000; Schmidt and Ferger, 2001; Yasuda *et al.*, 2008) and ATP depletion (Cassarino *et al.*, 1999). MPP^+

can also occupy monoamine vesicles, which can displace DA in the vesicles and induce auto-oxidation and free radical formation (reviewed by Dauer and Przedborski, 2003). The action of MPP^+ selectively destroys the dopaminergic neuron due to its affinity for the vesicular monoamine transporter (VMAT) and DAT (Xu *et al.*, 2005). The primary action of MPP^+ neurotoxicity is most likely to induce mitochondrial dysfunction. As secondary effects, increased DA auto-oxidation, neuronal activation of NADPH-oxidase and NOS, and microglial activation occur (reviewed by Singer and Ramsay, 1990). Differing doses, routines and durations of MPTP administrations may induce different severities of damage in the dopaminergic system. For example, neuronal loss was less severe in mice with acute MPTP administration (less than 50% dopaminergic neuron loss), while in the chronic MPTP/probenecid model, 70% of dopaminergic neurons and 90% of DA neurotransmitters were lost, which is similar to effects observed in PD patients (Meredith *et al.*, 2008). Therefore, the acute and chronic administrations of MPTP can be selected to set up different MPTP models to reflect different stages of PD.

1.5.3.2 The application of models

There are several *in vitro* and *in vivo* models that exist for PD research (reviewed by Schapira, 2002). In the present study, three models were chosen to investigate the mechanism of oxidative stress in PD. One was an *in vitro* model that investigates the oxidative stress mechanisms in PD at the cellular level. The other two models were animal models, including both an acute and a chronic model. MPTP was applied in different forms, including MPP^+, MPTP and MPTP/probenecid, respectively. As the neurotoxin MPTP is converted into its effective form, MPP^+ in the brain, MPP^+ is usually employed to create *in*

vitro models (Schapira, 2002). Probenecid which can prolong the effect of drugs was added with MPTP administration to create the chronic animal PD models. These animal models were used to investigate the oxidative stress mechanisms at the different stages of PD and at cellular and systemic levels.

(1) MPP^+-induced cellular model of PD

Rodent cells are genetically different from human cells. Thus, an immediate advantage would be gained by utilizing human-derived cells. It is known that human SH-SY5Y neuroblastoma cells are suitable as an *in vitro* model for PD research (reviewed by Naoi and Maruyama, 1999). Firstly, aside from being human-derived, these cells can be cultured in large quantities, thereby providing high reproducibility rates. Secondly, compared to human primary neurons, SH-SY5Y cells are a good model for long-term experiments, as they can survive for several generations, are easy to culture, and are inexpensive. Mature neuronal phenotypes can be achieved by chemically inducing differentiation with retinoic acid (RA) and 12-O-tetradecanoyl-13-phorbol acetate (TPA) combined with serum or growth factors. After TPA treatment, SH-SY5Y cells are further differentiated morphologically into neuronal cells. The combination of the RA and TPA treatments enhances the DA transport system and makes the cells more sensitive to MPP^+ (Presgraves *et al.,* 2004). Thus, RA/TPA-differentiated SH-SY5Y cells are considered to be a valid *in vitro* model.

However, the cellular model also has some limitations. In cell culture, it is difficult to mimic the interaction of various cell types, such as neurons and glial cells, which occur *in*

vivo. It also cannot be used to test the effect on the different brain regions and the pathways of the neurotoxic effects, and so cannot fully mimic the human brain systems.

(2) MPTP-induced acute model of mouse brain slices

Brain slice cultures have been used for direct studies of neurodegenerative mechanisms. A study with brain slices may reflect the appropriate changes of acute environmental insult and drug abuse (Notterpek *et al.*, 1993). Environmental toxin and drug abuse can trigger both acute and chronic neurodegeneration, such as those caused by dieldrin and 1-methyl-4-phenyl-4-propionoxypiperidine, desmethylprodine (MPPP), depending on the exposure duration (Kitazawa *et al.*, 2001). It is very important to understand the entire progression of PD. To choose the therapy for preventing and effectively treating PD, the pathogenesis in the early stage of PD also needs to be investigated. Thus, in this study, brain slices incubated with MPP^+ were created as an acute model of PD to study the mechanism by which acute neurotoxins induced neurodegenerative processes, which may be similar to the early stages of PD.

However, the limitation of this model is that it only reflects the acute effects of MPP^+, and not the chronic effects. In addition, although brain slices contain many structures in the brain, it is still not representative of an entire brain system.

(3) Chronic mouse model with MPTP/probenecid

Currently, the MPTP animal model is considered to be the most valid model for studying the neuropathological and neurochemical changes of PD. Because PD is a progressive

disease, acute and sub-chronic MPTP administrations, which may reflect some changes in earlier stages of PD, cannot mimic similar changes to those found in the late stage PD. To overcome this problem, a model created by the chronic administrations of MPTP with an adjuvant, a drug called probenecid, could mimic a progression similar to that in PD, since probenecid can block the rapid clearance of MPTP and its metabolites into the urine. Several studies have demonstrated that this model represents many pathological hallmarks, as well as motor disability, which are similar to those observed in PD patients (Meredith *et al.*, 2008). For example, chronic MPTP/probenecid significantly reduces the number of neurons in the SNpc (Meredith *et al.*, 2008). This is advantageous over acute or sub-chronic MPTP mouse models, in which less than 50% of the SNpc dopaminergic neurons are damaged, and the striatal DA level was depleted by only about 59% one day after MPTP insult, whereas the DA level was recovered to 28% from day 3 to 2 weeks after MPTP administration, due to the DA compensatory mechanism. However, 3 weeks after chronic MPTP/probenecid treatment, 70% of the DA neurons were lost in the SNpc, striatal DA levels in the mouse brains were reduced by 90–93% within one week and by 70–80% of the total at 3 to 24 weeks after MPTP/probenecid treatment (Meredith *et al.*, 2008). Similar findings in PD patients (at the late stage of PD) have shown at the onset of PD symptoms. 60% of the DA neurons were lost in the SNpc, and 70% or more of the DA concentration was depleted in the striatum (Hornykiewicz, 1975; reviewed by Dauer and Przedborski, 2003). Therefore, this model of MPTP with probenecid injection in the mouse is considered to be suitable for studying the pathology of PD and neuroprotective therapies.

Although this model can study the chronic effect of the neurotoxin in the entire brain system, it still has its limitations. It is an animal model, which has different species features compared to humans. MPTP/probenecid administrations may not produce effects that completely reflect all of the pathological mechanism in humans. However, it is still the most valuable model employed prior to pre-clinical trials. This problem, however, would appear in any animal model and is not unique to the MPTP/probenecid model.

Overall, after the comparison of the current PD models introduced above, and considering that MPTP can specifically damage the DA system, cause oxidative stress and is more convenient to set up despite its limitations, the MPTP model is the most suitable for studying the cellular and systemic mechanisms of PD, and may be a good model to reflect the different stages of PD. Thus, the oxidative stress mechanism of PD will be studied in these three models: the cellular model, brain slice model, and chronic MPTP/probenecid model.

1.6 The treatments of PD

1.6.1 Present treatments

The current treatments for PD fall into three categories. First, most medical treatments for PD are based on restoring the loss of DA production by using L-DOPA, a DA precursor that can increase DA, DA agonists to increase DA levels, or inhibitors of DA degradation (MAO-B inhibitors, COMT-inhibitors) to decrease DA metabolism (reviewed by Olanow *et al.*, 2001; reviewed by Rascol *et al.*, 2002). Second, anti-cholinergic drugs and N-

methyl-D-aspartic acid (NMDA) antagonists (amantadine) that block glutamates are used to treat PD patients. Third, the deep brain stimulation, which modulates dysfunctional basal ganglia circuits to protect dopaminergic transmission, is frequently utilized (Koller *et al.*, 1999; reviewed by Olanow, 2002). While medical and surgical therapies are able to reduce the symptoms of PD, these treatments cannot delay or stop the progression of the disease because they cannot stop dopaminergic neuron degeneration or the associated pathologies. Thus, although they help patients in the acute phase, current therapies cause adverse side effects and may even induce oxidative stress. They can increase DA content at the beginning, whereas an excess amount of DA can cause auto-oxidation and lead to the production of DA-quinone, which can cause neuronal injury, as mentioned in Section 1.4.4.2 (Chapter 1). Eventually, they would still be unable to stop DA neurodegeneration (reviewed by Lledó, 2000; Xia *et al.*, 2001). Thus, to develop a drug or natural product that can effectively improve the symptoms of PD and protect neurons, with or without side effects, is an urgent requirement for the treatment of PD patients. For example, natural products or drugs that can reduce oxidants, enhance the anti-oxidative system and protect DA neurons would be extremely valuable for preventing and treating PD.

1.6.2 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) have been demonstrated to act as multifunctional antioxidants (Richard *et al.*, 2008). A fatty acid is a carboxylic acid, often with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated. PUFA are a group of fatty acids which contain more than one double bond (Richard *et al.*, 2008). Essential fatty acids (EFA) are a group of PUFA that can be only consumed in the form of

dietary triglycerides. After digestion in the intestine, EFA are absorbed and transported by lipoproteins into the blood, and subsequently transferred into different tissues, including the brain, the retina, and the heart.

As shown in Figure 1.4, EFA can undergo cellular β-oxidation to provide ATP to cells. EFA can also undergo esterification into cellular lipids, including triglycerides, cholesterol esters, and phospholipids, all of which can release free fatty acids later by enzymatic/hydrolytic processes for subsequent metabolism. The phospholipids formed by EFA are very important for maintaining both the structural integrity and critical cellular functioning of membranes throughout the body and the brain. Dietary EFA present as linoleic acid (LA) and alpha-linolenic acid (α-LA) are activated to become high-energy forms known as fatty-acyl CoA. Fatty-acyl CoA can be converted into longer-chain fatty acids. Then these longer-chain fatty acids transformed into other polyunsaturated products, such as EPA, docosahexaenoic acid (DHA), and arachidonic acid (AA), as derived by a series of desaturation plus elongation reactions which are particularly prevalent in the liver, and to a lesser extent, in other tissues.

1.6.3 n-3 fatty acids and n-6 fatty acids

Omega (n)-3 and n-6 fatty acids are two groups of EFA in PUFA. N-3 fatty acids are a family of PUFA that have a final carbon-carbon double bond in the n-3 position. Important n-3 fatty acids are: α-LA, EPA, docosapentaenoic acid (DPA) and DHA. Alpha-LA is the precursor of EPA, DPA and DHA (Figure 1.5). However, very limited conversion of α-LA to EPA and DHA occurs. In addition, EPA is the precursor of DPA and DHA, and dietary

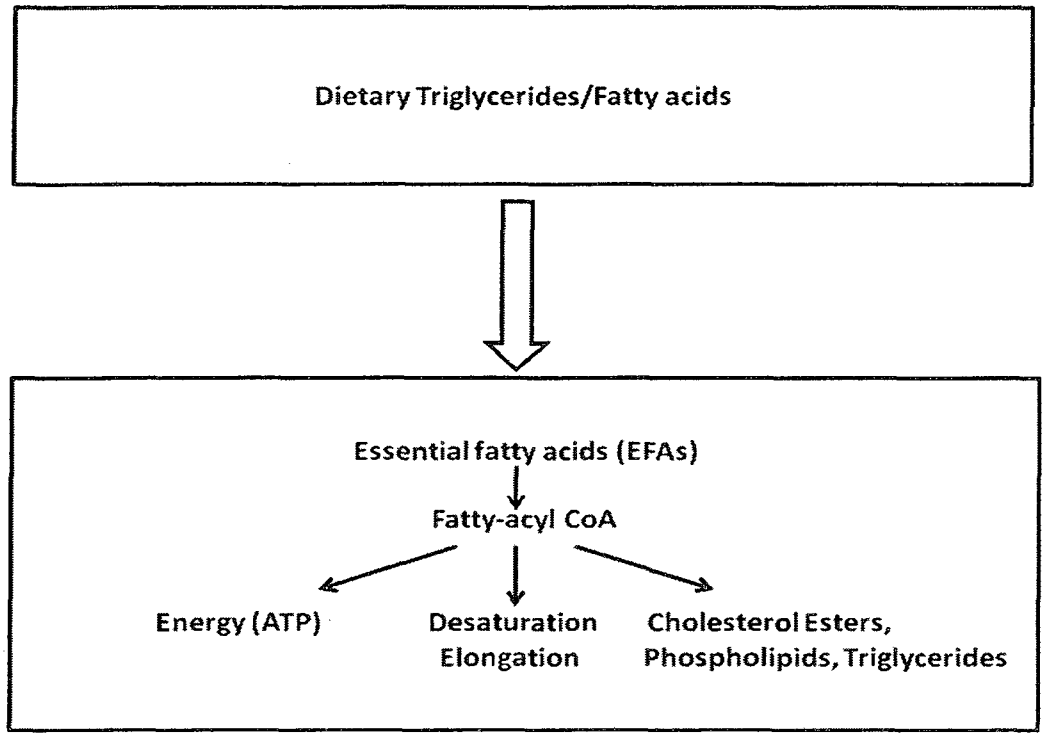

Figure 1.4. The EFA metabolism: Dietary EFA can undergo cellular β-oxidation to provide ATP to the cells, or undergo esterification into cellular lipids, including triglycerides, cholesterol esters, and phospholipids, which may be released as free fatty acids by enzymatic/hydrolytic processes for subsequent metabolism. They can also become more polyunsaturated products via desaturation and elongation (adapted from http://dhaomega3.org/index.php by Qingjia Meng).

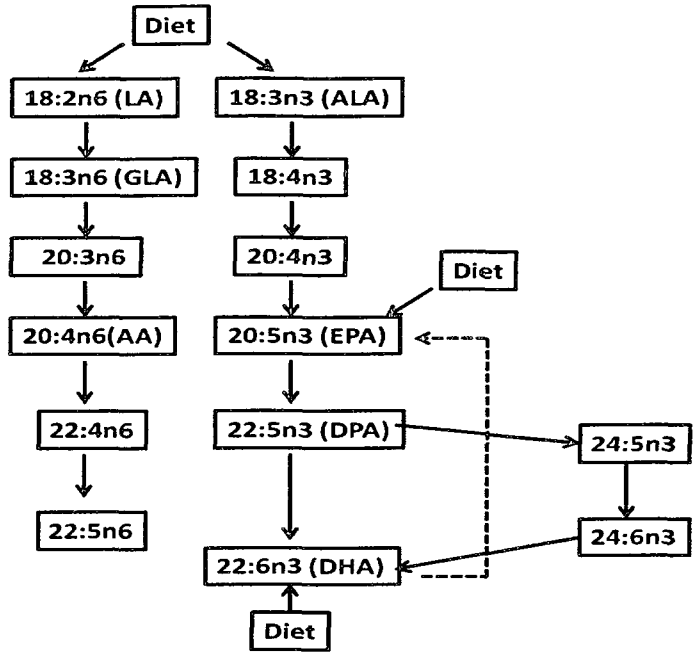

Figure 1.5. PUFA desaturation, elongation, and retroconversion: The precursors of n-3 and n-6 fatty acids are LA and α-LA, respectively. LA and α-LA can become other PUFA via desaturation and elongation (adapted from http://dhaomega3.org/index.php by Qingjia Meng).

DHA has the potential to undergo reverse metabolism back to EPA (reviewed by Brenna *et al.,* 2009). N–6 fatty acids are a family of unsaturated fatty acids, with carbon–carbon double bond in the n–6 position. Important essential n–6 fatty acids are: LA, gamma-linolenic acid (GLA), and AA (Figure 1.5). The n-6 AA, a product formed from the desaturation and elongation of LA, has very high concentrations in human tissues and cells (reviewed by Whelan, 2008).

1.6.4 n-3 fatty acids and cell function

N-3 fatty acids, one component of the biological bilayers of the neuronal cell membrane, have been reported to be potent neuroprotectors (reviewed by Das, 2000). The n-3 fatty acids significantly affect numerous functions of cell membranes. The carbon-carbon double bonds of n-3 fatty acids, with their high electron density and chains, are very flexible and can change conformational states, enabling the membrane to become more fluid (reviewed by Larsson *et al.*, 2004). Among these fatty acids, DHA (with six double bonds) and EPA (with five double bonds) are the best fatty acids for improving membrane fluidity. The more fluidity a membrane gains, the more efficient its biochemical performance would become. Because fluidity affects the physical properties of biological membranes, makes lipid and protein cooperation more diverse, and enhances communication, it can modulate protein function and trafficking, and vesicle budding and fusion (reviewed by Schmitz and Ecker, 2008). Therefore, n-3 fatty acids may promote the activation of various membrane-bound enzymes, transporters, and receptors. N-3 fatty acids not only modulate proteins, but many of them can also exert effects on protein function through altering gene expression. N-3 fatty acids can switch many different genes on and off by binding to the peroxisome

proliferator-activated receptors (PPAR). These effects suggest that having adequate levels of n-3 fatty acids in membrane systems is critical to cell survival, growth and renewal functions (Okuyama *et al.*, 2008; Richard *et al.*, 2008; Schmitz and Ecker, 2008).

1.6.5 n-3 fatty acids and inflammation

Many studies have demonstrated that n-3 fatty acids have strong anti-inflammatory functions. Both n-3 and n-6 fatty acids can modulate inflammation, but in most conditions they do so in opposite directions. Most of the mediators formed from EPA and DHA are anti-inflammatory, whereas AA can produce inflammatory responses through several immune mediators, as described in Figure 1.6 (Bagga *et al.*, 2003). The difference between n-3 and n-6 fatty acids in the modulation of the immune system stems from their different roles in eicosanoid production, because prostaglandin E3 (PGE3) and leukotriene B5 (LTB5) produced from EPA are anti-inflammatory mediators, and PGE2 and LTB 4 produced from AA are pro-inflammatory mediators.

Furthermore, n-3 fatty acids can directly inhibit the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Schmitz and Ecker, 2008), a transcription factor that plays a significant role in many inflammatory signaling pathways. NF-κB is essential for regulating the gene expression of molecules, such as several cytokines (e.g. interleukin (IL)-1, IL-2, IL-6, IL-12, TNF-α), chemokines (e.g., IL-8, MIP-1α, MCP1), adhesion molecules (e.g. ICAM, VCAM and E-selectin) and inducible enzymes (e.g. iNOS and COX-2), which are important for adaptive immune responses (reviewed by Ghosh and Karin, 2002).

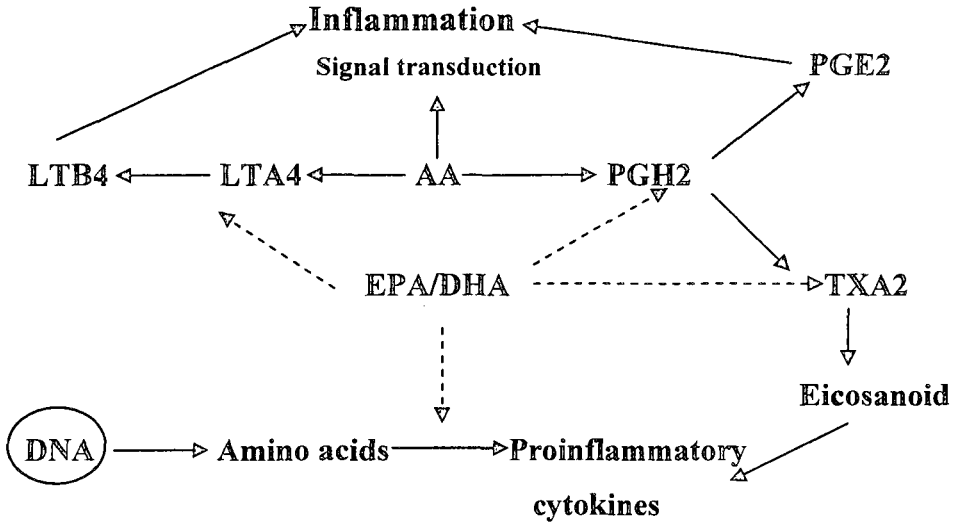

Figure 1.6. The relationship between n-3 and n-6 fatty acids and inflammation: n-3 (EPA) and n-6 (AA) are either precursors of EPA or eicosanoids. The solid arrows indicate synthesis and dashed indicate inhibition. LT: leukotriene; AA: arachidonic acid; PG: prostaglandin; TX: throboxane; EPA: eicosapentaenoic acid (adapted from James et al., 2000 by Qingjia Meng).

1.6.6 n-3 fatty acids and oxidative stress

The susceptibility of fatty acids to oxidation is thought to be directly related to their degree of unsaturation and also to their environment. Among the many fatty acids, DHA is the most oxidizable fatty acid, and palmitic acid is the least oxidizable. It has been suggested that n-3 fatty acids, including EPA and DHA, are easily oxidized in the bulk phase or dissolved in organic solvents, since they have many bis-allylic hydrogen atoms. However, in their phospholipid forms, their oxidative stability depends on the degree of fatty acid unsaturation. In an aqueous system, such as those in the cell membrane and in cell membrane interfaces, fatty acids are stable against oxidation. Evidence has shown that n-3 fatty acids have an anti-oxidative function rather than an oxidative function (reviewed by Okuyama *et al.*, 2008; Richard *et al.*, 2008). The reason could be that n-3 fatty acids affect the oxidant/antioxidant status of the brain by stabilizing cell membrane structure (Ozyurt *et al.*, 2007). For example, one study demonstrated that the n-3 fatty acids DHA and EPA can reduce oxidative stress and may play a positive regulatory role in the synthesis of antioxidant enzymes such as SOD and GSHpx (Li *et al.*, 2006). In addition, DHA and EPA may form more hydrophobic interfaces between the phospholipid bilayers, which might prevent membrane entry of the hydrophilic H_2O_2 molecule. Evidence also supports the theory that n-3 fatty acids appear to operate generally against endogenously produced ROS and can protect membrane proteins (proteins inside and outside organelles for eukaryotes) from being damaged by oxidants (Okuyama *et al.*, 2008).

1.6.7 n-3 fatty acids and PD

The n-3 fatty acids act as functional food defenses against neurodegenerative diseases (reviewed by Calon and Cole, 2007). From experimental models and human postmortem tissues, a significant decrease of PUFA levels and an increased level of MDA, an indicator of lipid peroxidation, have been reported in the SN of PD patients when compared to other brain regions (Dexter *et al.*, 1989). N-3 fatty acids may correspondingly increase DA levels without producing oxidants due to their antioxidant function, and prevent PD in the *in vivo* model (Yehuda, 2002). A study showed that a long-term dietary deficiency in n-3 PUFA induces a significant reduction in the amount of DA and DA D2 receptors, specifically in the frontal cortex, due to the functional relation between the frontal cortex and the limbic system (Le Moal and Simon, 1991; Delion, 1996; King, 1997). In two recent prospective studies, people who ate a diet high in saturated fatty acids and low in unsaturated fatty acids showed a higher risk of developing PD (Chen *et al.*, 2003; de Lau *et al.*, 2005). In an MPTP animal model of PD, a high n-3 PUFA dietary intake exerted neuroprotective actions by maintaining the DA level in spite of the subacute MPTP-induced decrease in DA concentrations (Bousquet *et al.*, 2008). In addition, as Sections 1.6.5 and 1.6.6 have highlighted, n-3 fatty acids can modulate inflammation and oxidative stress, which are both involved in the pathology of PD. It is becoming more and more convincing that n-3 fatty acids may reduce the risk of PD development.

Although it is reported that n-3 fatty acids may prevent PD, the correlation between these fatty acids and PD-induced oxidative stress, and the interrelation between the biosynthesis

and metabolism of these fatty acids in the brain and oxidative stress are unknown. The present study sought to evaluate the influence of n-3 fatty acids on MPTP models of PD and determine its relationship to oxidative stress.

1.6.8 EPA

EPA is an n-3 fatty acid referred to as 20:5(n-3), and a major component of fish oil. Currently, fish oil products are popularly sold in the nutrient market. EPA, as one of the n-3 fatty acids, was reported to have the potential to prevent PD (Bousquet *et al.*, 2008). However, research evidence supporting EPA modulation of the oxidative and anti-oxidative systems in PD is scarce. For the past 10 years, our research team has investigated the effect of EPA on neurodegenerative diseases, including AD and neuroinflammation models. It is reported that both dietary EPA and DHA have anti-inflammatory properties, but EPA has been shown to be more potent (Sierra *et al.*, 2008). As mentioned before, several studies have suggested that similar to other n-3 fatty acids, EPA may have the potential to attenuate oxidative stress in PD (Chen *et al.*, 2003; de Lau *et al.*, 2005), increase the activity of the antioxidant enzyme SOD, and prevent H_2O_2 from entering the cells in the plasma membrane, preventing cellular attack (Nishida *et al.*, 2006; Sohma *et al.*, 2007). It was also found that EPA attenuates iNOS and NO levels in stimulated microglia (Moon *et al.*, 2007). EPA can also modulate inflammation, cognitive impairment, and behavioral disorder (Song *et al.*, 2003; 2008; 2009). Other benefits of EPA have been introduced along with other n-3 fatty acid in section 1.6. Therefore, EPA was chosen as a new test therapy in the present study to determine its effects on attenuating oxidative stress in PD models.

1.7 Hypothesis and objective

It is well known that oxidative stress is one of the major contributors to the etiology of PD, but so far, the mechanisms of oxidants and antioxidants in PD remain unclear. Whether MPTP affects the n-3 and the n-6 fatty acid profile through oxidative stress is also unknown. As an antioxidant, EPA has the potential to attenuate oxidative stress in PD; however, there has been no study revealing the antioxidant mechanism of EPA in PD.

The primary objectives of this study were to investigate 1) the interrelation between the oxidative and anti-oxidative systems in the MPTP/MPP^+-induced PD models, and 2) to determine whether EPA can attenuate oxidative stress in three PD models induced by MPTP/MPP^+: a cellular model, brain slice model, and an *in vivo* model. To reach this aim, the following research aspects were investigated:

1. The effects of MPTP or MPP^+ on oxidative stress mechanisms at different levels (mitochondrial founction, oxidant production, antioxidant protection and lipid metabolism)
2. The beneficial effects of EPA alone on oxidative stress mechanisms at the levels mentioned above.
3. The therapeutic mechanism of EPA in the treatment of MPTP/MPP^+-induced changes in oxidative and anti-oxidative systems.

The general hypotheses for the study as a whole were (1) MPTP/MPP^+ may increase oxidant production and destroy the balance between the oxidative and anti-oxidative systems, which may contribute to the reduction of mitochondrial founction in the cellular

model of PD and contribute to the changes in the brain fatty acid profile in the animal models; (2) EPA treatment alone may have no effect on oxidants and antioxidants in the normal condition, or it may promote cell functions and increase brain n-3 fatty acids and (3) the EPA treatment may attenuate MPTP/MPP^+-induced changes.

The thesis is divided into four chapters. Chapter 1 is a general introduction of the whole thesis. Chapter 2 is a study to explore the interrelationship between the oxidative and anti-oxidative systems in a cellular model of PD and effects of n-3 fatty acid EPA treatment, and Chapter 3 is a study to investigate efficacy of EPA against MPTP effects on oxidative stress and the fatty acid profile in brain slices model and in a chronic C57BL/6 mouse model. In Chapter 4, the general discussion of these three models in different research contexts is provided.

Chapter 2

An Exploration of the Interrelationship between the Oxidative and Anti-oxidative Systems in a Cellular Model of Parkinson's Disease: Effects of Omega-3 Fatty Acid EPA Treatment

2.1 Introduction

PD is caused by the loss of dopaminergic neurons in the SN and the striatum (Antoniades and Barker, 2008). Recently, increasing evidence suggests that inflammation and oxidative stress may contribute to the neuropathogenesis of PD. However, the primary causes of cell death remain controversial.

Many studies in PD patients have shown that oxidative stress may play an important role in the etiology of PD (reviewed by Jenner and Olanow, 2006). Indeed, pro-oxidant enzymes such as NADPH-oxidase, which produces oxidant $O_2^{\cdot}$, are up-regulated in the SNpc of PD patients (Wu *et al.*, 2003). Higher expression of the NOS enzyme, which triggers NO production, was found in the nigrostriatal region and basal ganglia in postmortem PD brains (Eve *et al.*, 1998). Conversely, decreased levels of the major antioxidant GSH occur in the brain of PD patients (Zoccarato *et al.*, 2005; Zeevalk *et al.*, 2007). This evidence indicates that PD is strongly related to oxidative stress.

Oxidative stress may result from an imbalance between the production of oxidants and antioxidants (reviewed by Mena *et al.*, 2008). Oxidants are mainly composed of ROS and RNS. ROS are triggered by NADPH-oxidase and include $O_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$ (a product of the reaction between H_2O_2 and $O_2^{\cdot}$). RNS are triggered by NOS and include NO and $ONOO^{\cdot}$ (a product of the reaction between NO with $O_2^{\cdot}$). Antioxidant and antioxidant enzymes are mainly composed of SOD, GSH, GSHpx and catalase, and their functions have already been introduced in Chapter 1, section 1.4.2 (reviewed by Özben, 1998).

To study the oxidative mechanisms of PD and evaluate new treatments, MPTP has been commonly used as a dopaminergic neurotoxin, since it can induce severe parkinsonian-like syndromes with the loss of nigrostriatal dopaminergic cells in animals and humans (Lotharius and O'Malley, 2000). The active metabolite of MPTP, MPP^+, is used in many *in vitro* studies, and the general findings are that MPP^+ can induce oxidative stress and apoptosis in neurons and catecholaminergic cell lines, such as SH-SY5Y cells (Cassarino *et al.*, 1997). For example, an increase in H_2O_2 production in MPP^+-treated SH-SY5Y has been reported (Kalivendi *et al.*, 2003). MPP^+ has also been shown to increase oxidant NO levels in SH-SY5Y cells, as seen by imaging a fluorescent adduct produced by the reaction of NO and 4-amino-5-methylamino-2'7'-difluorofluorescein (DAF-FM) (Dennis and Bennett, 2003). As well, several studies on non-neuronal, neuronal, and glial cell lines, such as PC12 (Ibi *et al.*, 2006), SH-SY5Y (Nikolova *et al.*, 2005), and GT1-7 (Schneider *et al.*, 2003) showed that the pro-oxidant enzyme NADPH oxidase was increased by MPP^+. However, results from studies with MPTP and MPP^+ are not always consistent with one another. First, one study showed that the toxicity of MPP^+ may contribute to an increase in nNOS (O'Byrne and Tipton, 2002). By contrast, another study reported that MPP^+ treatment causes a time-dependent decrease in nNOS activity in cells that overexpressed nNOS (Shang *et al.*, 2005). Second, regarding SH-SY5Y cells, it was reported that activities of the antioxidants SOD and catalase were inhibited by MPP^+ (Jung *et al.*, 2007). However, another study showed that the antioxidant enzyme activities of SOD, catalase and GSHpx were increased rather than decreased in the SH-SY5Y cells after exposure to MPP^+ (Cassarino *et al.*, 1997). From the above mentioned evidence, it can be seen that inconsistent results occur, and the interrelationship between the oxidative and anti-

oxidative systems remains unclear. Thus, the first aim of the present study was to further understand the interrelationship between oxidative and anti-oxidative systems in the MPP^+-treated cellular model by studying oxidants, antioxidants and their enzymes.

As mentioned in Chapter 1, Section 1.6.1, the efficacy of PD treatment to date is limited. Since inflammation and oxidative stress may contribute to the etiology of PD, a drug or natural product to reduce these effects may be beneficial to PD patients. N-3 fatty acids, one member of the polyunsaturated PUFA family and an important component of neuronal cell membranes, have been reported to be a potent neuroprotector (reviewed by Das, 2000) and have the potential to reduce the risk of developing PD (Bousquet *et al.*, 2008). The supporting evidence includes: 1) From experimental models and human postmortem tissues, a significant decrease of PUFA levels and an increased level of lipid peroxidation have been found in the SNpc when compared to other brain regions of PD patients, as evidenced by increased levels of MDA (Dexter *et al.*, 1989); 2) N-3 fatty acids affect the oxidant/antioxidant status of the brain by stabilizing the membranous structure of cells (Ozyurt *et al.*, 2007); 3) EPA, one of the n-3 fatty acids, may alleviate oxidative stress, since it prevents H_2O_2 from entering the cells through the plasma membrane (Nishida *et al.*, 2006) and can also attenuate iNOS and NO levels in stimulated microglia (Moon *et al.*, 2007) and 4) EPA and DHA were found to promote levels of GSH, an antioxidant, and antioxidant enzyme activities were significantly increased after EPA and DHA treatments (Kim and Chung, 2007). EPA was also found to increase SOD mRNA gene expression in rat hepatocytes (Sohma, 2007). However, to date, few studies have evaluated the effect of EPA as an antioxidant in brain cells, and no study has evaluated the effect of EPA on oxidative stress in an MPP^+-induced PD model or PD patients. Thus, the second aim of the

present study was to evaluate the effects of EPA treatment on MPP^+-induced changes in oxidative and anti-oxidative systems.

The two aims mentioned above generated two hypotheses. Firstly, MPP^+ would increase the production of oxidants and pro-oxidant enzymes, but decrease the protection of antioxidants. Secondly, EPA would attenuate MPP^+-induced oxidative stress. To test these hypotheses, SH-SY5Y cells treated with MPP^+ were used as an *in vitro* model of PD. Cell viability was tested by MTT assay. The production of the oxidants H_2O_2 and NO were measured by 2',7' dichlorofluorescein diacetate (DCF-DA) and 4,5-diaminofluorescein diacetate (DAF-2DA). The mRNA expressions of the pro-oxidant enzymes, NOS and NADPH-oxidase, were measured by quantitative PCR. The protein expression of NADPH-oxidase was measured by Western blot. mRNA expressions of the antioxidant enzymes SOD, catalase, and GSHpx were measured by quantitative PCR. Antioxidant GSH levels were quantified using a GSH assay kit.

2.2 Methods

2.2.1 Chemicals: MPP^+ iodide (Sigma, D048) was prepared in deuterium-depleted water (ddH_2O) and aliquoted as 100 mM frozen at -80 °C. Working solutions were prepared by diluting stock MPP^+ in cell culture medium. EPA-sodium salt (Sigma, E6627) was prepared in cell culture medium in concentrations of 10 mM and also frozen at -80 °C.

2.2.2 Cell culture procedures

SH-SY5Y cells, kindly donated by Dr. John Bradley's group at the National Research Council Institute for Nutrisciences and Health, were cultured in a 100 ml flask with DME/F-12 1:1 (Hyclone, SH30023.01) medium containing 1% penicillin-Streptomycin (Hyclone, SV30010), 1% Sodium Pyruvate (Invitrogen, 11360-070), 2% Sodium Bicarbonate (Hyclone, SH30033.01), 1% Non-essential Amino Acid Solution (Hyclone, SH30238.01) and 10% fetal bovine serum (FBS) (Hyclone, SH30071.03), and incubated with 5% CO_2 and 95% air at 37°C. When the cells reached 80% confluence, the media was removed, and 1X Phosphate Buffered Saline (PBS) was added. The cells were detached by treatment with 2.5 ml 0.25% trypsin and 2.5 ml PBS for 5 minutes at 37°C in an incubator. The trypsin (Sigma, 59429C) was deactivated by adding 10 ml of medium (FBS inactivates the trypsin). Cells were centrifuged at 200 x g for 5 minutes and the pellet was then resuspended in culture medium. The cells were stained with Trypan Blue (Sigma, T8154) and counted with a hemocytometer. Based on the cell concentration counted, all of the cells were seeded into 96-well plates at a density of 2.0×10^5 cells/ml or 6-well plates at a density of 5.0×10^5 cells/ml.

2.2.3 Experimental treatments

Before applying any treatment, SH-SY5Y cells were differentiated to dopaminergic neuron-like cells. The cells were treated with 10 μM RA (Sigma, R2625) on the second day after being seeded. Two days later, the media was replaced with fresh media containing the same amount of RA. Another 2 days later, the media was replaced with fresh media containing 80 nM phorbol TPA (Sigma, P8139). After 3 days of TPA differentiation, the

morphology of the cells became neuron-like with long dendrites, in accordance with established morphological criteria (Lanciotti *et al.*, 1991; Presgraves *et al.*, 2004). Upon reaching full differentiation, the cells were treated with MPP^+ 0.1 μM to 100 μM (0.1, 1, 10, 50 and 100 μM) or EPA 0.1 μM to 100 μM (0.1, 1, 10, 50 and 100 μM) for 48 to 96 hours (The incubation times will be provided in the results section in each experiment). Before carrying out the other assays, the cell viability of the fully differentiated SH-SY5Y cells with different doses of MPP^+ or EPA was studied by MTT assay. Based on the results, the optimal dose of EPA that can induce the greatest increase in cell viability and the optimal doses of MPP^+ that can best reduce cell viability were chosen, and then combined to test EPA protective effects on MPP^+-treated cells. Because MPP^+ at 50 and 100 μM induced similar suppressions on cell viability, for some parameters, both doses were used; while for some, one dose was used after the most effective dose was determined. Finally, the optimal doses of EPA and MPP^+ were combined to test EPA neuroprotective effect on MPP^+-treated SH-SY5Y cells.

2.2.4 MTT assay

Mitochondrial dehydrogenase cleaves the tetrazolium salt MTT (Thiazolyl Blue Tetrazolium Bromide) into the formazan product and was used as a measure of mitochondrial activity in a colorimetric assay (Marks *et al.*, 1992). The MTT test can be useful to quantify the activation level of cells via mitochondrial dehydrogenase (Gerlier and Thomasset, 1986). General mitochondrial activity was determined by assaying the reduction of MTT into formazan (Vukmanović and Zamoyska, 1991). SH-SY5Y cells seeded in 96 well-plates (2.0×10^5 cells/ml) were used in this assay. Following

experimental treatment, MTT (Sigma, M2128) was added to the culture media at a final concentration of 0.5 mg/ml and incubated for 4 hours at 37 °C. Subsequently, cells and dye crystals were solubilized with 200 µl dimethyl sulfoxide (DMSO) (Sigma, D8418) and absorbances were measured at 570 nm using a microplate assay reader (Molecular Devices, CA).

2.2.5 H_2O_2 assay

The cells were seeded in a 96-well plate (2.0×10^5 cells/ml) for this assay. Following experimental treatment, the level of intracellular ROS was quantified by DCF-DA, as described by Bass *et al.* (1983). DCF-DA, a non-fluorescent compound, was deacetylated into the cells to 2',7'-dichlorofluorescein (DCF) by H_2O_2. The cells were incubated with 100 µM DCF-DA (dissolved in DMSO) (Sigma, 3584) for 30 minutes at 37°C and washed three times with PBS (pH 7.4). With PBS, the relative levels of fluorescence in the cells were quantified using excitation and emission wavelengths of 485 and 538 nm, respectively, in a microplate assay reader. The measured fluorescence values were expressed as a percentage of the fluorescence in control cells (Kim *et al.*, 2007).

2.2.6 NO assay

Following the experimental treatment procedures mentioned above, DAF-2DA (Cayman, CAS 205391-02-2) was used to detect NO. DAF-2DA is a cell-permeable derivative of DAF-2, and is used as a sensitive fluorescent indicator for the detection and bioimaging of NO. After the cells absorbed DAF-2DA, DAF-2DA was transformed into the less cell-permeable DAF-2 by cellular esterases, and prevented a loss of signal due to diffusion of

the molecule into the cell. SH-SY5Y cells were seeded in 96 well plates (2.0×10^5 cells/ml). Following experimental treatment and incubation, the culture medium was removed and the cells were washed in DMEM without phenol red and 10% FBS, and then placed in 200 ml DMEM medium without phenol red and 10% FBS. DAF-2DA was added at a final concentration of 1μM and incubated for 30 minutes at 37°C. Following incubation, the concentration of NO in the cells was tested by Fluoscan using excitation and emission wavelengths of 485 and 538 nm, respectively (Dennis and Bennett, 2003).

2.2.7 RNA extraction

Cells seeded in 6-well plates (5.0×10^5 cells/ml) were utilized to conduct RNA extraction. RNA extraction was conducted as per manufacturer's instructions. Medium was removed, and then one ml of TRI-reagent was added to each well, and the cells were thoroughly lysed in TRI-reagent by pipetting. After a 5 minute incubation period, the cell suspension was collected in eppendorf tubes. 200 μl of chloroform was added to each tube. The tubes were thoroughly shaken for 15 seconds and incubated for 3 minutes. After being centrifuged for 15 minutes at 12000 x g, the RNA in the upper layer of the tube was removed. To each tube was added 500 μl of isopropyl alcohol, followed by an incubation period of 10 minutes. After being centrifuged for 10 minutes, the supernatant was removed and 1 ml of 70% ethanol was added to each tube. The tubes were then vortexed and centrifuged at 7500 x g for 5 minutes. The supernatant was then removed and 50 μl diethylpyrocarbonate (DEPC)-treated water was added into each tube. The extracted RNA was stored at -80°C.

2.2.8 cDNA synthesis

QuantiTect Reverse Transcription (Qiangen, 205311) was carried out to synthesize cDNA from the collected RNA. The RNA concentration was adjusted to 1 μg/μl by DEPC-treated H_2O. One μl template RNA with 2 μl genomic DNA wipeout buffer (containing a DNase) and 7 x 11 μl RNase-free water were added into the tubes. The template RNA mixture was incubated for 2 minutes at 42°C and immediately placed on ice after incubation. The template RNA mixture was added into each tube, which contained a reverse-transcription master mix, and then incubated for 15 minutes at 42°C, and an additional 3 minutes at 95°C to inactivate the Quantiscript Reverse Transcriptase. Each completed reverse transcription reaction was followed directly with quantitative PCR, or stored at -20°C.

2.2.9 Quantitative PCR

A QuantiTect SYBR Green PCR kit was used for quantitative PCR. All PCR reactions were performed on a Rotor Gene 6000 Quantitative PCR machine. For each PCR run, a master mixture was prepared on ice with primers, DNAse-RNAse-free water and cDNA. 15 minutes at 95°C with 20°C/sec incubation was applied to activate Hotstar Taq DNA polymerase. Following step cycling 15sec at 94°C with 20°C/sec was first used to denature the cDNA. The cDNA was annealed for 20-30 sec at 50-60°C after denaturation, and then extended for 10-13 sec at 72°C with 2°C/sec. The reaction included 55 cycles. After 55 cycles, a melting curve was generated to determine primer specificity and identity, and each melting curve of the primer is presented in an appendix. The specific forward/reverse primers used were: GSHpx1: 5′-ATGTGTGCTGCTCGGCTCTC-3′/5′-TGCTGGGACAGCAGGGTTTC-3′; SOD2: 5′- ACCTTTCTCAGTAGCGGCAA-3′/5′-

TGTGTCACCTTGTCAGGGAA-3′; Catalase: 5′-AGGTTTGGCCTCACAAGGAC-3′/5′-GCGGTAGGGACAGTTCACAG-3′; nNOS: 5′- AATGCTACGTCAGCCTTGCT-3′/5′-TACAGCACTGTGGCCTTGAC-3′; $P47^{phox}$: 5′-CGAGAAGAGTTCGGGAACAG-3′/5′-TCCAGATAGGATGCAGGGAC -3′; and β-actin reference: 5′-GTCGTACCACTGGCATTGTG-3′/5′-CTCTCAGCTGTGGTGGTGAA -3′. The ΔΔCT method was choosen to test the comparative cycle time (Bookout *et al.*, 2006). In each individual sample, the ΔΔCT method was used to calculate the relative quantitation of each target gene normalized with β-actin levels, and then the target gene expression was compared between the treated group and the control ($\Delta Ct = Ct_{Target\ gene} - Ct_{\beta\text{-}actin}$; $\Delta\Delta Ct = \Delta Ct_{treated\ group} - \Delta Ct_{control}$; relative amount of target gene = $2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). Beta-actin, a housekeeping gene, is commonly used as a reference gene (Mori *et al.*, 2008). It has been shown that due to the aggravation of oxidative damage, the gene expressions of cysteine dioxygenase 1 and thioredoxin interacting protein, which are related to the toxicity of MPP^+ and anti-oxidative mechanisms, were suppressed under the MPP^+ treatment from 16 to 48 hours (Wang *et al.*, 2008). In another study, the gene expression of complex I (subunit 4) was reduced after 72 hours of MPP^+ treatment. The change in complex I function has been known to be directly related to the oxidative damage in the cells (Conn *et al.*, 2001). In addition, the apoptosis biomarker, an increase in caspase 3 gene expression, has been found after 96 hours of exposure to MPP^+ (Zhang *et al.*, 2007). As well, because the significant reduction of cell viability was found 48-96 hours after MPP^+ incubation by our team, the treatment time, 48 hours, was selected to be used in the gene expression experiment.

2.2.10 Western blotting

2.2.10.1 Protein collection:

Cells seeded in 6-well plates (5.0×10^5 cells/ml) were collected for Western blot. After collecting and washing twice with PBS, cells were collected in eppendorf tubes and centrifuged at 16,000 xg for 10 minutes. All of the supernatant was removed from the sample. The supernatant was then stored at -80°C or lysed. A radio-immunoprecipitation assay (RIPA) buffer was used for cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins' immunoreactivity and biological activity, which included 20 mM Tris, 150 mM NaCl, 1% Nonidet P-40; 0.5% Sodium Deoxycholate, 1 mM EDTA, and 0.1% SDS as adjusted to pH 7.5 with HCl, and stored at 4-6°C. Protease inhibitor (Sigma, Canada) was also prepared as 20 g tissue/ml. Cells were then lysed by sonication (Misonix Inc, XL2000) with 60 µl lysis buffer and protease inhibitor at power level 2. Lysates were centrifuged at 10,000 x g for 10 minutes at 4°C.

2.2.10.2 Sample preparation:

To denature the protein, lysed samples were diluted in 2 x loading buffer (1:1) and heated for less than 5 minutes at 95°C. The denatured samples were then put on ice. After cooling down, samples were vortexed and spun down. 10-20 µl of the sample (20-40 µg) of protein was loaded into the well of the gel. Electrophoresis took place for 1 hour in total, starting at 80V and increasing to 120V when the samples reached the separation gel.

2.2.10.3 Gel transfer:

Polyvinylidene fluoride (PVDF) membranes (Millipore, MA, ISEQ00010) were adjusted to fit the gel but be slightly larger than the filters, immersed in methanol for 10 minutes, and then immersed in transfer buffer for another 10 minutes at 4°C. Filter paper (Millipore, MA, IBFP0813C) was also adjusted and immersed for 10 minutes in the transfer buffer at 4°C. The gels were adjusted and immersed alone in the transfer buffer at 4°C. Semi-dry gel transfer was conducted on the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad laboratories, Canada, 170-3940). The gel sandwich was composed, from bottom to top, of a bottom electrode, a sheet of filter paper, the PVDF membrane, the acrylamide gel, another sheet of filter paper, and a top electrode. Bubbles were avoided, especially between the membrane and gel, since this may affect transfer efficiency. Transfer was started at 15V for 60 minutes. After transfer, the membranes were adjusted to the sample area and rinsed with clean ddH_2O.

2.2.10.4 Immunoblotting

Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Actin rabbit polyclonal IgG 200 µg/ml (SC-7210) and the NADPH-oxidase $p47^{phox}$ rabbit polyclonal IgG 200 µg/ml (SC-14015) were used as primary antibodies. The primary antibodies were diluted to 1µg/ml in the blocking buffer. Donkey anti-rabbit IgG-HRP (SC-2077) 400 µg/ml was used as secondary antibody and diluted as 1:500 - 1:10000 in the blocking buffer for immunoblotting. Membrane incubation was processed in petri dishes for the entire experiment. The membrane was washed with Tris-Buffered Saline Tween-20 (TBST) for 5 minutes, and 5% non-fat milk block solution was

applied to block the membrane for a minimum of 2 hours. Next, membrane incubation underwent another TBST wash for 5 minutes at room temperature on a plate shaker. The membrane was then incubated in the primary antibodies for at least 2 hours at room temperature, or 4°C overnight, followed by three TBST washes for 20, 20, and 10 minutes, respectively. The membrane was then incubated in the secondary antibody for 1 hour at room temperature, followed by another three TBST washes for 20, 20 and 10 minutes, respectively. After washing, the membrane was again washed briefly with TBST, then dried with Kimwipes and imaged.

2.2.10.5 Imaging:

Supersignal West-Pico Chemiluminescent Substrate (Pierce, IL, 34080) was applied to improve the signal. The substrate components were mixed at a ratio of 1:1, added onto the dry membrane, and then wrapped with plastic membrane. The membrane was placed at the centre of the tray of the Bio-Rad molecular imager Gel Doc XR system (Bio-Rad laboratories, 170-8170) and placed in the dark for appropriate durations (usually 1 minute).

2.2.10.6 Data analyses:

The images of actin and the NADPH-oxidase $p47^{phox}$ were quantified by software Image-J (version 1.4). Images were captured, scanned, and analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Background staining was removed from each group. To sample protein content, data were divided by actin to normalize the NADPH-oxidase $p47^{phox}$ protein expression. Treatment values were normalized to their respective control band intensity and

the results were then expressed as a fold-change over control levels.

2.2.11 GSH assay

Cells were collected with PBS, transferred into a microcentrifuge tube and centrifuged at 600 x g until they formed a packed pellet. The supernatant was then removed. 5% 5-Sulfosalicylic acid solution was then added to the tubes at three times the volume of the pellet and vortexed. The suspension was frozen and thawed twice in liquid nitrogen and a 37°C water bath, and left for 5 minutes at 4°C. The extract was centrifuged at 10,000 x g for 10 minutes. The supernatant was kept at 4°C as a sample. A GSH assay kit was used to determine GSH concentration. The measurement of GSH used a kinetic assay (Sigma, CS0260) in which catalytic amounts (nmoles) of GSH caused a continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) into 5-thionitrobenzoic acid, and the GSSG formed was recycled by GSH reductase and NADPH. The product, 5-thionitrobenzoic acid, was assayed colorimetrically at 412 nm using a microplate reader.

Calculations

GSH standard solutions were utilized to determine the standard curve and calculate the ΔA412/minutes equivalent to 1 nmole of reduced GSH per well. The amount of GSH in the unknown sample was obtained from the following formula:

GSH concentration (nmoles/ml sample) = ΔA412/minutes (sample) x dil/ (ΔA412/minutes (1 nmole) x vol sample)

The following are a list of the notations and the calculations:

ΔA412/minutes (sample) = slope generated by sample (after subtracting the values generated by the blank reaction); ΔA412/minutes (1 nmole) = slope calculated from standard curve for 1 nmole of GSH; dil = dilution factor of original sample; vol = volume of sample in the reaction in ml

2.2.12 Statistical analysis

Data were analyzed using GraphPad Prism 4.0 and presented as mean ± SEM. When EPA or MPP^+ was used alone as a single factor, a one-way ANOVA was used. Following an ANOVA showing a significant factor effect, the *post-hoc* Bonferroni test was utilized to analyze the different dose responses. Each group with different treatment was compared to the control group. In the MPP^+ and EPA combined experiment, there were two factors involved, the EPA treatment factor and the MPP^+ treatment factor. Two-way ANOVA was used to analyze the effects of MPP^+ and EPA as two factors on the different parameters (MPP^+: 0 μM, 50 μM or/and 100 μM, EPA: 0 μM and 100 μM). Following a two-way ANOVA showing a significant factor effect, the *post-hoc* Bonferroni test was carried out to analyze group differences. With the *post-hoc* test, the results were compared at different levels. The most interesting three levels were presented: the result of the MPP^+ group was compared to the control, the result of the EPA treatment group was compared to the control, and the EPA and MPP^+ combination group was compared to the group treated with MPP^+ alone. P values less than 0.05 were considered to be statistically significant. Outliers determined by the box plot test in the statistical analyses were removed from the experiments. Any removed outliers are shown in the test. If no outlier was shown, it indicated that no outlier was removed from the statistical analyses. The number of

replicates in each experiment was presented in its figure test. The variations of the number of replicates among the groups were accounted for by the following reasons. Each well had been checked according to the morphological criteria (Lanciotti *et al.*, 1991; Presgraves *et al.*, 2004a), and the differentiated cells in any wells not qualified were removed before the assay. Any samples unable to be detected or any samples with technical problems were removed before the statistical analysis.

2.3 Results

2.3.1 The effect of MPP^+ and EPA on mitochondrial dehydrogenase activity

Mitochondrial dehydrogenase activity was measured by MTT assay after an incubation period of 48 hours. MPP^+ at doses ranging from 0.1 to 100 μM induced an effect on mitochondrial dehydrogenase activity ($F_{5, 66} = 56.17$, $p<0.001$, compared to the control, by one-way ANOVA). The *post-hoc* test showed that MPP^+ induced a dose-dependent reduction in the mitochondrial dehydrogenase activity as low as 1 μM ($t = 3.265$, $p<0.05$) and as high as 100 μM ($t = 12.26$, $p<0.001$) compared to the control (Figure 2.1A). Aside from the lack of statistically significant differences between the 50 and 100 μM doses ($t = 1.567$, $p>0.05$), all other doses were significantly different from one another (control vs. MPP^+ 1 μM: $t = 3.265$, $p<0.05$; MPP^+ 1 μM vs.10 μM, $t = 4.213$, $p<0.01$; MPP^+ 10 μM vs. MPP^+ 50 μM: $t = 3.215$, $p<0.05$).

After treating SH-SY5Y cells with 0.1 to 100 μM EPA in 96 well plates, an enhanced effect on mitochondrial dehydrogenase activity was observed ($F_{5, 66} = 7.796$, $p<0.001$, by one-

way ANOVA), and a *post-hoc* test indicated that EPA at 50 ($t = 3.519$, $p<0.05$) and 100 μM ($t = 5.667$, $p<0.001$) significantly increased mitochondrial dehydrogenase activity as compared to the control (Figure 2.1B).

To evaluate the protective effects of EPA on MPP^+-induced changes, the following 6 groups were included: control, MPP^+ at 50 μM alone, MPP^+ at 100 μM alone, EPA alone, MPP^+ at 50 μM with EPA, and MPP^+ at 100 μM with EPA. It was found that MPP^+ as a main factor significantly affected mitochondrial dehydrogenase activity ($F2, 61 = 51.87$, $p<0.001$), and EPA as a main factor significantly affected mitochondrial dehydrogenase activity ($F1, 61 = 93.98$, $p<0.001$) as well. There was no interaction between EPA and MPP^+ ($F2, 61 = 1.686$, $p = 0.1938$). The *post-hoc* test showed that MPP^+ alone at both 50 μM ($t = 6.062$, $p<0.001$) and 100 μM doses ($t = 5.018$, $p<0.001$) significantly suppressed mitochondrial dehydrogenase activity when compared to the control. EPA at 100 μM alone significantly increased mitochondrial dehydrogenase activity when compared to the control ($t = 7.645$, $p<0.001$). After culturing MPP^+ at 50 μM and EPA at 100 μM together, mitochondrial dehydrogenase activity was significantly higher than that in the group treated with MPP^+ at 50 μM alone ($t = 7.312$, $p<0.001$). EPA at 100 μM combined with MPP^+ at 100 μM also significantly increased mitochondrial dehydrogenase activity when compared to MPP^+ at 100 μM alone ($t = 4.562$, $p<0.001$) (Figure 2.1C) (Cell number has been tested in our group. It was found the changes of cell number did not affect the results).

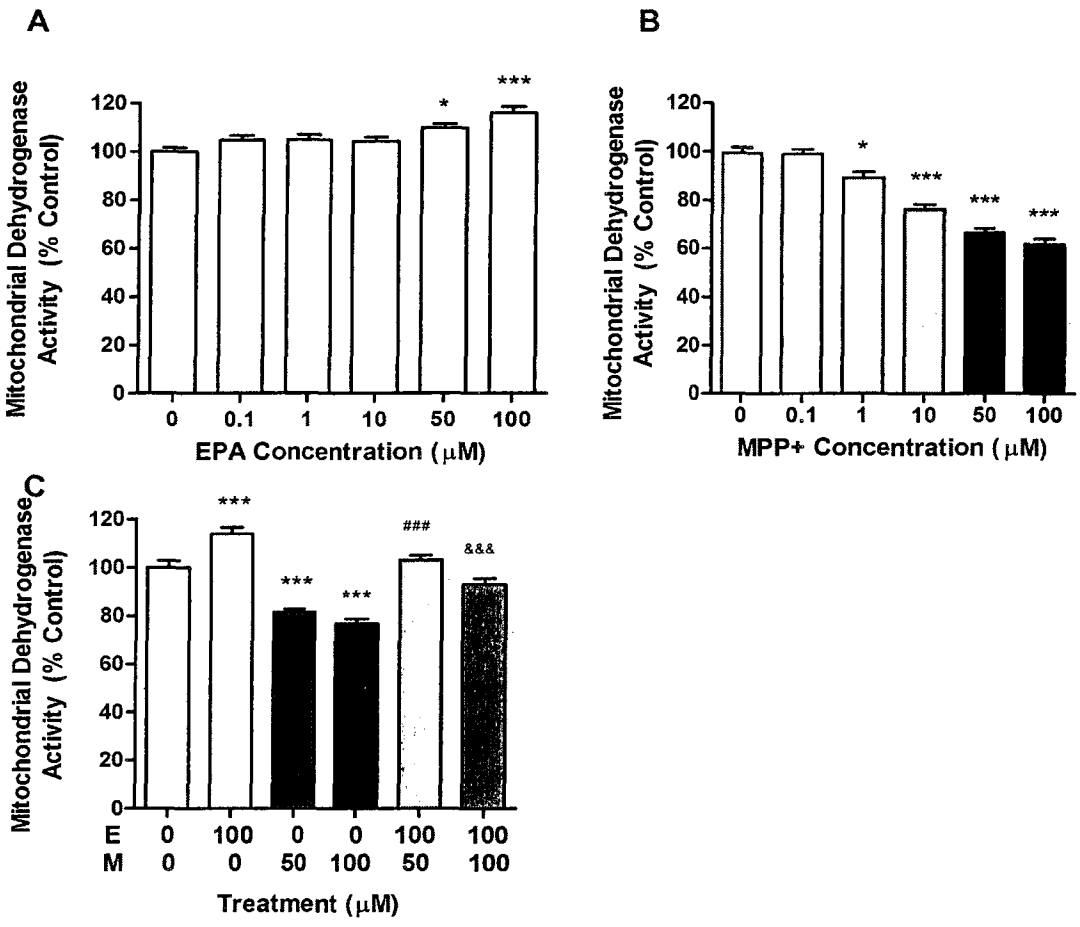

Figure 2.1.The effect of MPP^+ and EPA on mitochondrial dehydrogenase activity: Mitochondrial dehydrogenase activity of fully differentiated SH-SY5Y cells were measured by MTT assay with the following for 2 days: (A) MPP^+ (at 0.1 to 100 μM) (n = 12), (B) EPA (at 0.1 to 100 μM) (n = 12) and (C) MPP^+ and EPA combined (C: n = 11; E: n = 11; M50: n = 12; M100: n = 12; EM50: n = 12 and EM100: n = 9). Data are presented as mean ± SEM and expressed as the percentage of control. * $p<0.05$ as compared to the control, *** $p<0.001$ as compared to the control, ### $p<0.001$ as compared to the MPP^+ at 50 μM, &&& $p<0.001$ compared to the MPP^+ at 100 μM treated group. C, non-treated control; E, EPA100 μM; M50, MPP^+ 50 μM; M100, MPP^+ 100 μM; EM50, MPP^+ 50 μM and EPA 100 μM and EM100, MPP^+ 100 μM and EPA 100 μM.

2.3.2 The effect of MPP^+ and EPA on ROS production

To determine the effect of MPP^+ treatment on H_2O_2 production and test the anti-oxidative function of EPA, DCF-DA fluorescence was used as an indicator of H_2O_2 production by SH-SY5Y cells. It was found that MPP^+ had no effect after 48 hours (data not shown), thus, 96 hours was chosen. In response to different doses of MPP^+ (0.1 to 100 μM), one-way ANOVA showed that MPP^+ significantly increased H_2O_2 production ($F_{5, 63} = 7.238$, $p<0.001$). The *post-hoc* test indicated that MPP^+ at 0.1 ($t = 4.081$, $p<0.05$), 1 ($t = 3.075$, $p<0.05$), and 50 μM ($t = 2.977$, $p<0.05$) significantly up-regulated H_2O_2 production (Figure 2.2A). Because MPP^+ at 50 μM significantly decreased mitochondrial dehydrogenase activity and also increased the H_2O_2 production, this dose was selected to be combined with EPA in the combined experiment. In the experiment testing EPA different doses, EPA had no significant effect on H_2O_2 production ($F_{5, 62} = 0.2598$, $p = 0.9361$) (Figure 2.2B).

In the experiment with EPA and MPP^+combination, there were 4 groups: control, MPP^+ at 50 μM alone, EPA alone, and MPP^+ at 50 μM with EPA. The EPA treatment factor did not significantly affect H_2O_2 production ($F_{1, 39} = 0.5387$, $p = 0.4672$, by two way ANOVA), whereas the MPP^+ treatment factor significantly affected H_2O_2 production ($F_{1, 39} = 6.108$, $p<0.05$, by two-way ANOVA). There was also an interaction between MPP^+ and EPA ($F_{1, 39} = 12.32$, $p<0.01$, by two-way ANOVA), indicating a mutually dependent effect between EPA and MPP^+. The *post-hoc* test showed that MPP^+ at 50 μM increased the H_2O_2 production from SH-SY5Y cells when compared to the control ($t = 4.092$, $p<0.001$). EPA alone had no effect on H_2O_2 production of SH-SY5Y cells compared to the control.

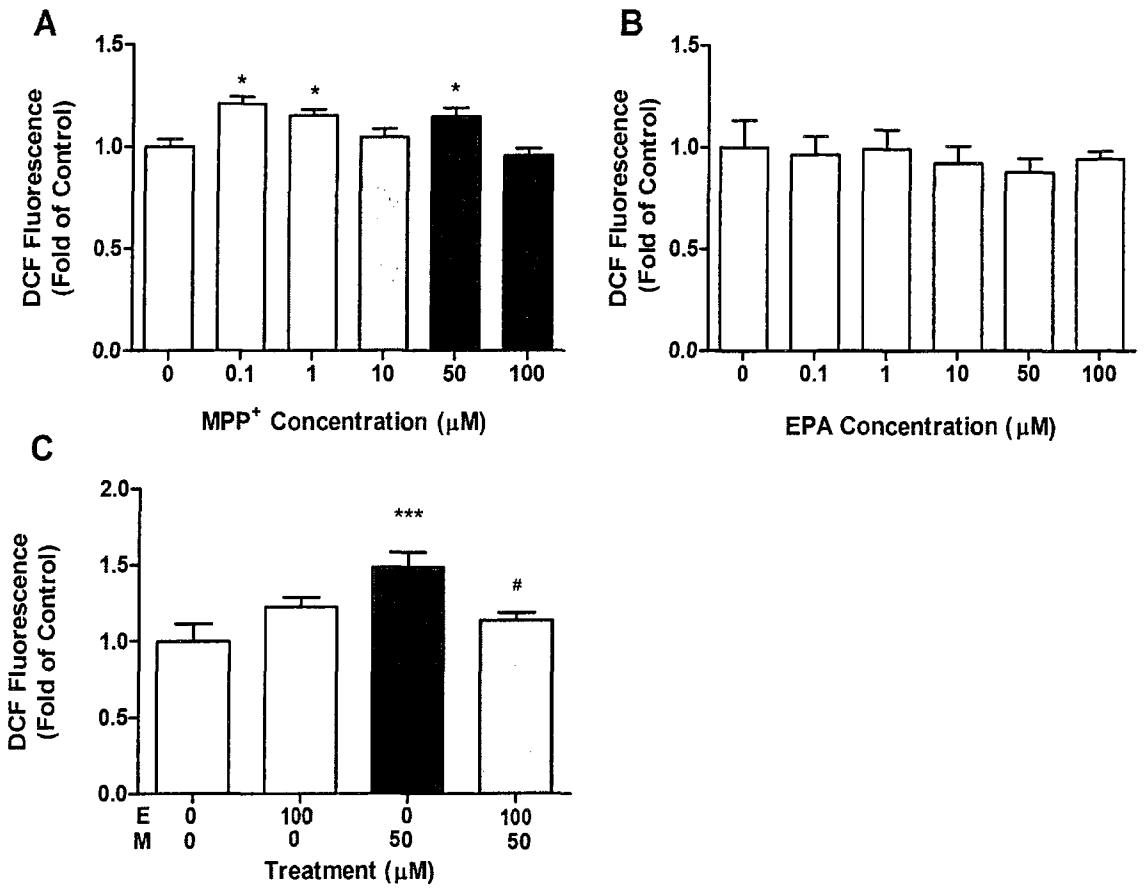

Figure2.2.The effect of MPP^+ and EPA on ROS production: The production of ROS was measured by DCF-DA with the following treatments in SH-SY5Y cells for 3 days: (A) MPP^+ (at 0.1, 1, 10, 50, 100 μM) (C: n = 13, M0.1: n = 10, M1: n = 11, M10: n = 13, M50 = 11, M100: n = 11), (B) EPA (at 0.1, 1, 10, 50, 100 μM) (C: n = 12, E0.1 = 12, E1, 10, 50, 100: n = 11) and (C) EPA and MPP^+ combined (E: n = 12; C, M50: n = 10; EM50: n = 11). The data are presented as mean ± SEM and presented as the fold of control. * $p<0.05$ as compared to the control, *** $p<0.001$ as compared to the control, # $p<0.05$ as compared to the MPP^+ at 50 μM, && $p<0.01$ as compared to the MPP^+ at 100 μM. C, non-treated control; E, EPA100 μM; M50, MPP^+ 50 μM and EM50, MPP^+ 50 μM and EPA 100 μM.

However, in the MPP^+ combined with EPA group, the effect of MPP^+ at 50 μM was down-regulated by EPA when compared to the MPP^+ group ($t = 2.972$, $p<0.05$) (Figure 2.2C).

2.3.3 The effect of MPP^+ and EPA on RNS production

To determine the effect of MPP^+ treatment on RNS production, and to test EPA antioxidant function, NO production in SH-SY5Y cells was studied. It was found that MPP^+ had no effect on NO production after culturing cells with MPP^+ for 48 hours (data not shown); thus, 96 hours was chosen. In the experiment using MPP^+ at different doses, a significant effect of the MPP^+ treatment was found ($F5, 58 = 7.687$, $p<0.001$, by one-way ANOVA). The *post-hoc* test showed that MPP^+ at 1 ($t = 3.819$, $p<0.01$), 10 ($t = 4.627$, $p<0.001$), and 100 μM ($t = 4.623$, $p<0.001$) significantly up-regulated NO production (Figure 2.3A). In the experiment using EPA different doses, a one-way ANOVA showed that EPA had no significant effect on NO production ($F5, 63 = 0.4155$, $p = 0.8363$) (Figure 2.3B).

To study the anti-oxidative effect of EPA, the experiment with EPA combined with MPP^+ contained 6 groups: control, MPP^+ at 50 μM alone, MPP^+ at 100 μM alone, EPA alone, MPP^+ at 50 μM with EPA treatment, and MPP^+ at 100 μM with EPA treatment. A two-way ANOVA showed that the MPP^+ treatment factor significantly affected NO production from SH-SY5Y cells ($F2, 52 = 87.66$, $p<0.001$) but the EPA treatment factor

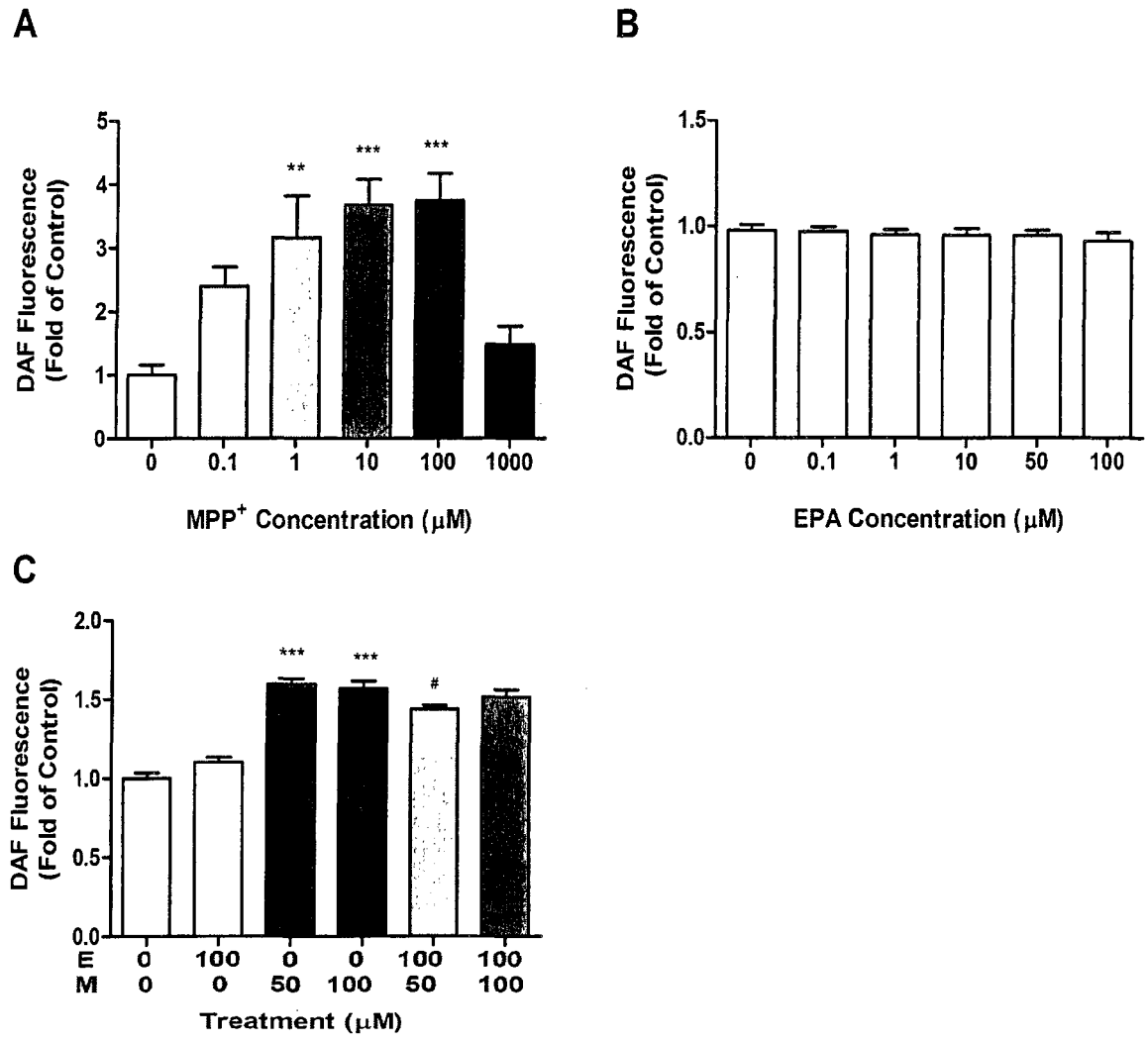

Figure 2.3.The effect of MPP^+ and EPA on RNS production: The production of RNS was measured by DAF-2DA in differentiated SH-SY5Y cells for 3 days with (A) MPP^+ (at 0.1, 1, 10, 100, 1000 μM) (C, M10: n = 11; M0.1, 100, 1000: n = 10; M1: n = 12), (B) EPA (at 0.1, 1, 10, 50, 100 μM) (C: n = 11; E0.1, 1 , 50, 100: n = 12; E10: n = 10), and (C) EPA and MPP^+ combined treatment (C: n = 8, E, M50, M100, EM50, EM100: n =10). The data are expressed as mean ± SEM as the fold of control. ** $p<0.01$ as compared to the control, *** $p<0.001$ as compared to the control, # $p<0.05$ as compared to the MPP^+ at 50 μM. C, non-treated control; E, EPA100 μM; M50, MPP^+ 50 μM; M100, MPP^+ 100 μM; EM50, MPP^+ 50 μM and EPA 100 μM and EM100, MPP^+ 100 μM and EPA 100 μM.

did not significantly affect NO production from SH-SY5Y cells ($F1, 52 = 1.274$, $p = 0.2643$). There was an interaction between EPA and MPP^+ ($F2, 52 = 5.325$, $p<0.01$, by two-way ANOVA). The *post-hoc* test showed that MPP^+ at 50 μM ($t = 9.760$, $p<0.001$) and 100 μM ($t = 9.143$, $p<0.001$) alone both increased NO production from SH-SY5Y cells when compared to the controls, but EPA alone had no effect on NO production when compared to the controls. In the combined experiment, the effect of MPP^+ at 50 μM was down-regulated by EPA ($t = 3.106$, $p<0.05$) when compared to the effect of MPP^+ at 50 μM, while EPA failed to attenuate the effect of MPP^+ at 100 μM when compared to the effect of MPP^+ at 100 μM (Figure 2.3 C).

2.3.4 The effect of MPP^+ and EPA on the expressions of pro-oxidant enzymes

To test the interrelationship between MPP^+ and EPA-induced changes in mRNA and protein expressions of pro-oxidant enzymes, quantitative PCR and Western blot were applied to monitor the changes in pro-oxidant enzymes. This quantitative PCR experiment contained 6 groups: control, MPP^+ at 50 μM alone, MPP^+ at 100 μM alone, EPA at alone, MPP^+ at 50 μM with EPA treatment, and MPP^+ at 100 μM with EPA treatment. Some extreme values were removed. The pro-oxidant enzyme $p47^{phox}$ (a major subunit of NADPH-oxidase) mRNA was monitored in the experiment. One outlier was removed from the100 μM MPP^+ group. The MPP^+ main factor significantly affected the $p47^{phox}$ gene expression ($F2, 29 = 22.090$, $p<0.001$ by two-way ANOVA), and the EPA main factor also significantly affected $p47^{phox}$ mRNA expression ($F1, 29 = 13.629$, $p<0.001$ by two-way ANOVA). There was an interaction between MPP^+ and EPA factors as well ($F2, 29 = 4.234$, $p<0.05$, by two-way ANOVA). The *post-hoc* analysis showed

that the $p47^{phox}$ mRNA expression was increased by MPP^+ at 50 μM ($t = 6.042$, $p<0.001$) and 100 μM ($t = 5.176$, $p<0.001$) to more than 2.5 fold of the control, and EPA alone had no effect on $p47^{phox}$ mRNA expression compared to the control. When EPA and MPP^+ were combined, EPA treatment significantly down-regulated the effect of MPP^+ at 50 μM and 100 μM compared to MPP^+ at 50 μM and 100 μM alone ($t = 2.877$, $p<0.05$ and $t = 3.629$, $p<0.01$, respectively) (Figure 2.4A).

The mRNA expression of another pro-oxidant enzyme, nNOS, was found to be significantly affected by the MPP^+ factor ($F2, 27 = 12.63$, $p<0.001$ by 2-way ANOVA), but not the EPA factor ($F1, 27 = 0.2316$, $p = 0.6342$). There was also an interaction between the MPP^+ and EPA factors ($F2, 27 = 4.942$, $p<0.05$ by two-way ANOVA). The *post-hoc* test showed that nNOS mRNA was significantly decreased by MPP^+ at 50 μM ($t = 5.033$, $p<0.001$) and 100 μM ($t = 5.158$, $p<0.001$) alone when compared to the control. However, EPA alone significantly decreased nNOS gene expression in SH-SY5Y cells when compared to the control ($t = 2.812$, $p<0.05$). When EPA and MPP^+ were co-cultured, EPA showed no significant effect on the MPP^+-induced reduction in the mRNA expression of nNOS compared to the group treated with MPP^+ alone (Figure 2.4B).

To test the protein expression of NADPH-oxidase, EPA was combined with MPP^+ to form four groups (one outlier has been removed from the control group): control, MPP^+ at 50 μM alone, EPA alone, and MPP^+ at 50 μM with EPA treatment. Results similar to its mRNA expression experiment were obtained. MPP^+ played a significant effect on

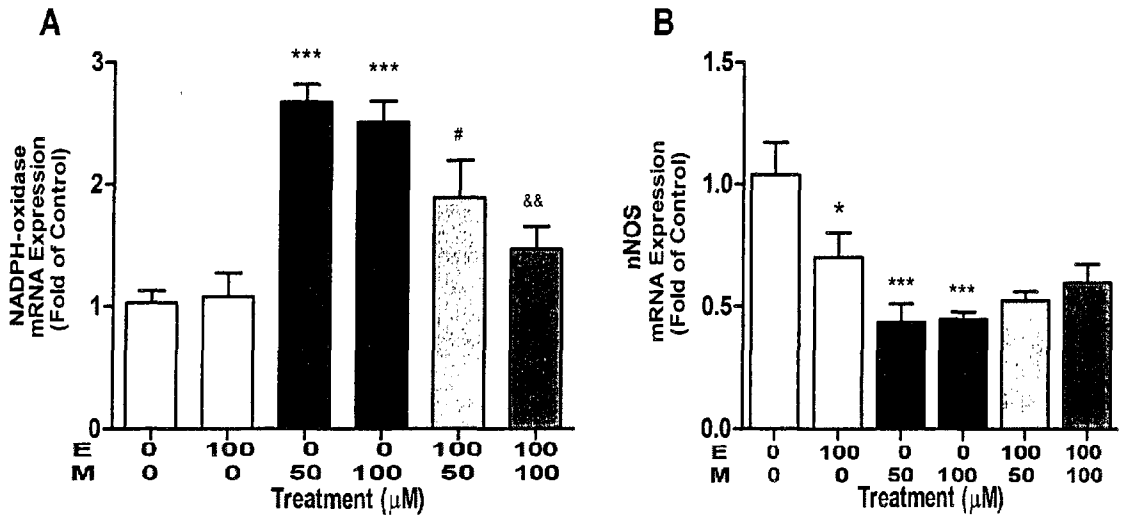

Figure 2.4.The effect of MPP^+ and EPA on mRNA expressions of the pro-oxidant enzymes: The effect of EPA and MPP^+ on mRNA expressions of the pro-oxidant enzymes in SH-SY5Y cells treated with the MPP^+ and EPA combined treatment for 2 days. (A) The NADPH-oxidase $p47^{phox}$ mRNA expression of SH-SY5Y cells (n = 6) and (B) the nNOS mRNA expression of SH-SY5Y cells. The results of PCR are expressed as mean ± SEM (C, M100, EM100: n = 6; E, M50, EM100: n = 5), expressed as the fold of control. ** $p<0.01$ as compared to the control, *** $p<0.001$ as compared to the control, # $p<0.05$, as compared to the MPP^+ at 50 μM, && $p<0.01$ as compared to the MPP^+ at 100 μM. C, non-treated control; E, EPA100 μM; M50, MPP^+ 50 μM; M100, MPP^+ 100 μM; EM50, MPP^+ 50 μM and EPA 100 μM and EM100, MPP^+ 100 μM and EPA 100 μM.

$p47^{phox}$ protein expression (F1, 15 = 12.95, $p<0.001$, by two-way ANOVA) and EPA also had a main effect on $p47^{phox}$ protein expression (F1, 15 = 9.871, $p<0.01$, by two-way ANOVA). There was a significant interaction between EPA and MPP^+ (F1, 15 = 9.871, $p<0.01$, by two-way ANOVA). The *post-hoc* showed that MPP^+ significantly up-regulated the NADPH-oxidase $p47^{phox}$ protein expression when compared to the control group (t = 4.631, $p<0.01$). EPA alone had no effect when compared to the control, but attenuated the effect of MPP^+ on $p47^{phox}$ protein expression when compared to the MPP^+ group (t = 6.118, $p<0.001$) (Figure 2.5). The best picture of the $p47^{phox}$ protein expression is shown in Figure 2.5.

2.3.5 The effect of MPP^+ and EPA on the gene expressions of antioxidant enzymes

Gene expressions of the antioxidant enzymes were tested in 6 groups: control, MPP^+ at 50 μM alone, MPP^+ at 100 μM alone, EPA alone, MPP^+ at 50 μM with EPA treatment, and MPP^+ at 100 μM with EPA treatment (48 hours). SOD was significantly affected by MPP^+ (F2, 57 = 16.46, $p<0.001$, by two-way ANOVA), but not by EPA (F1, 57 = 2.822, p = 0.0985), and there was no interaction between MPP^+ and EPA (F2, 57 = 0.6894, P = 0.5060). The *post-hoc* test showed that although the mRNA expression of SOD was increased by both doses of MPP^+ to more than twice the control level (MPP^+ 50 μM: t = 3.933, $p<0.001$; MPP^+ 100 μM: t = 4.492, $p<0.001$), EPA had no effect compared to the control, nor did it attenuate the effect of MPP^+(Figure 2.6A).

MPP^+ also had a major effect on catalase mRNA expression (F2, 57=10.35, $p<0.001$, by 2-way ANOVA), but EPA did not significantly change catalase mRNA expression (F1, 57 =

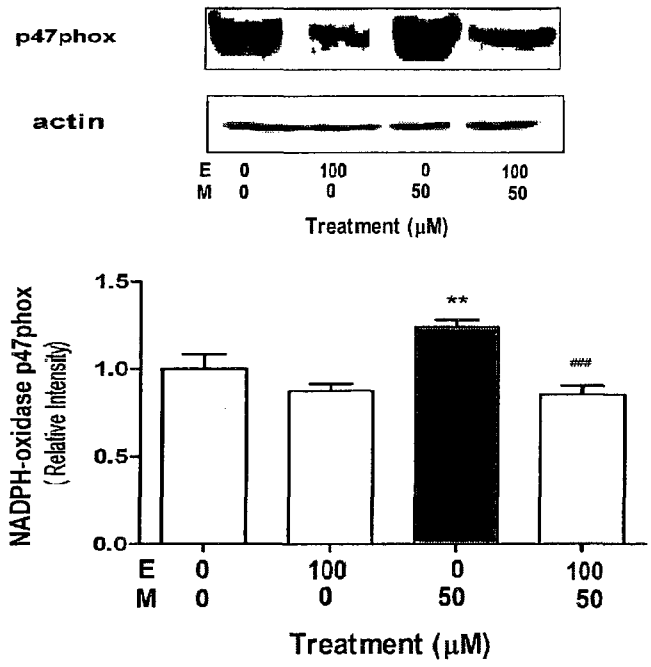

Figure 2.5.The effect of MPP^+ and EPA on protein expression of the NADPH-oxidase $p47^{phox}$: The results of Western blot are expressed as mean ± SEM (n = 5). Data were divided by actin to normalize the NADPH-oxidase p47phox protein expression. ** $p<0.01$ as compared to the MPP^+ at 50 μM, ### $p<0.001$ as compared to the MPP^+ at 50 μM (for 2 days). E, EPA; M, MPP^+.

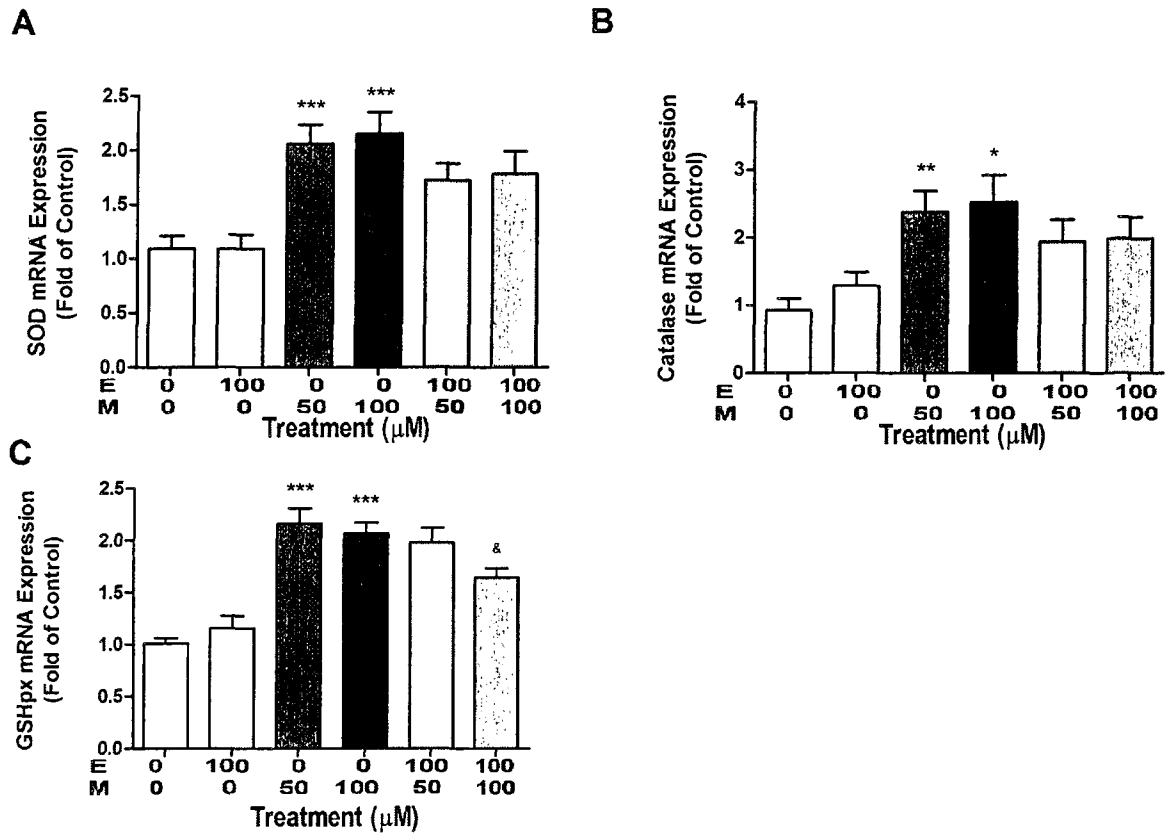

Figure 2.6.The effect of MPP^+ and EPA on the mRNA expressions of antioxidant enzymes: The bar chart is showing the mRNA expressions of antioxidant enzymes by quantitative PCR in EPA and MPP^+-treated SH-SY5Y cells for 2 days. (A) SOD mRNA expression (C, M100: n = 12; E: n = 11; M50, EM50, EM100=10), (B) catalase mRNA expression (C, E, EM50: n = 10; M50, M100, EM100: n = 11) and (C) GSHpx mRNA expression (C, E: n = 10; M50: n = 11; M100, EM50: n = 12; EM100: n = 9) were tested. The results are expressed as mean ± SEM and presented as the fold of control (n = 10). * $p<0.01$ as compared to the control, ** $p<0.01$ as compared to the control, & $p<0.01$ as compared to the MPP^+ at 100 μM. C, non-treated control; E, EPA 100 μM; M50, MPP^+ 50 μM; M100, MPP^+ 100 μM; EM50, MPP^+ 50 μM and EPA.

0.5797, p = 0.4495). There was no interaction between these two factors (F2, 57 = 0.1441, p = 0.8661). The *post-hoc* test showed that MPP^+ at 50 μM (t = 3.447, p<0.01) and 100 μM (t = 2.351, p<0.05) increased catalase mRNA expressions to two fold of the control. EPA treatment alone did not change the mRNA expression of catalase as compared to the control. In the group in which EPA was combined with MPP^+, EPA again did not present any attenuating effect when compared to the group treated with MPP^+ alone (Figure 2.6B).

In mRNA expression analysis of the third antioxidant enzyme, GSHpx, two-way ANOVA suggested that MPP^+ produced a significant main effect (F2, 58 = 39.24, p<0.001), but EPA did not (F1, 58 = 2.520, p = 0.1179). There was an interaction between the two factors that approaches statistical significance (F2, 58 = 2.843, p = 0.066). The *post-hoc* test showed that MPP^+ at 50 μM (t = 6.948, p<0.001) and 100 μM (t = 6.515, p<0.001) alone significantly up-regulated the mRNA expression of GSHpx when compared to the control, and EPA alone had no effect. EPA significantly reduced the effect of MPP^+ at 100 μM (t = 2.511, p<0.05), but not at 50 μM, as compared to the two MPP^+ alone treatment groups, respectively (Figure 2.6 C).

2.3.6 The effect of MPP^+ and EPA on GSH content

To quantify GSH concentrations in the cells, four groups were set up, including control, MPP^+ at 50 μM alone, EPA alone, MPP^+ at 50 μM with EPA treatment (48 hours). Two-way ANOVA indicated that MPP^+ produced a main effect on GSH levels in SH-SY5Y cells (F1, 36 = 24.75, p<0.001), but EPA had no effect on GSH levels (F1, 36 = 0.1392, p = 0.7112). There was no interaction between MPP^+ and EPA (F1, 36 = 0.1955, p = 0.6611).

The *post-hoc* test showed that MPP^+ at 50 μM alone significantly increased GSH levels in SH-SY5Y cells when compared to the control ($t = 3.205$, $p<0.01$). EPA alone had no effect when compared to the control, and in addition, EPA did not change the effect of MPP^+ in the combined group as compared to the MPP^+ alone (Figure 2.7).

2.4 Discussion

In this study it was found that MPP^+ alone, when compared to the control, significantly decreased mitochondrial dehydrogenase activity, increased ROS and RNS productions and enhanced the enzyme NADPH-oxidase $P47^{phox}$ mRNA and protein expressions; however, it decreased nNOS mRNA expressions. In addition, MPP^+ increased the mRNA expressions of the antioxidant enzymes SOD, catalase and GSHpx, as well as the level of the antioxidant GSH. EPA alone, when compared to the control, up-regulated mitochondrial dehydrogenase activity, and down-regulated nNOS mRNA expressions, but it had no significant effect on other parameters. However, compared to the group in which MPP^+ was administered alone, the group where EPA was combined with MPP^+ showed that EPA attenuated the effect of MPP^+ on mitochondrial dehydrogenase activity, ROS and RNS production, mRNA and protein expressions of NADPH-oxidase, and the mRNA expression of the antioxidant enzyme GSHpx. Whereas in the group with EPA and MPP^+ treatments, EPA had no significant effect on the mRNA expression of the pro-oxidant enzyme nNOS, or the antioxidant enzymes SOD and catalase when compared to the MPP^+-treated group. EPA did not attenuate the MPP^+-induced increase in antioxidant GSH levels either. The following discussions are arranged according to the order of results as they are presented.

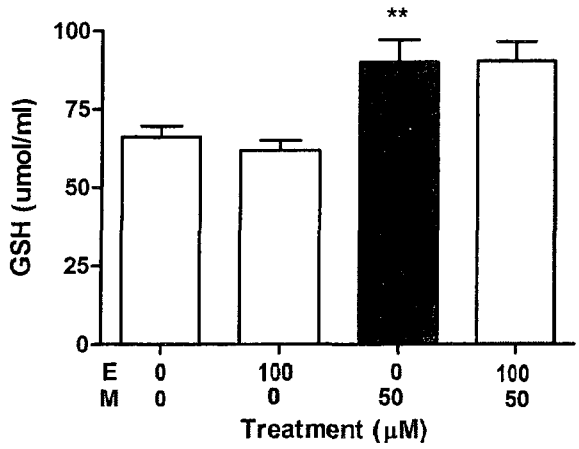

Figure 2.7.The effect of MPP^+ and EPA on the concentration of the antioxidant GSH: SH-SY5Y cells were treated with an EPA and MPP^+ combination for 2 days. The cells were collected after incubation. The GSH level in the cells was tested using a GSH assay kit, and results are expressed as mean ± SEM (μmol/ml) (n=10). ** $p<0.01$ as compared to the control. E, EPA; M, MPP^+.

2.4.1 The effect of MPP^+ on mitochondrial dehydrogenase activity, oxidant ROS and RNS production and pro-oxidant enzymes

RA/TPA differentiated SH-SY5Y cells are susceptible to MPP^+ cellular toxicity and these cells have been used as a model for studying the neuropathology and protective effects of drug in PD (Presgraves *et al.*, 2004). Indeed, in the present study, MPP^+ caused a reduction of mitochondrial dehydrogenase activity, as measured by MTT assays, which is in line with other reports that have used different cell models (Storch *et al.*, 2004; Su *et al.*, 2008; Yuan *et al.*, 2008). In addition, this finding again indicated that mitochondrial dysfunction is involved in MPP^+-induced toxicity (O'Callaghan *et al.*, 1990b). It is important to note here that the mitochondrial dehydrogenase activity tested by MTT assay cannot specifically reflect cell death. In our lab, it has already been shown that MPP^+ at doses of 50 and 100 μM and the incubation times of 48 and 96 hours cannot significantly induce cell death as determined by Trypan Blue (unpublished results). Thus, the results generated from the current study could reflect changes in cellular function and a possible involvement of oxidative and anti-oxidative systems after neurotoxin administration.

Oxidative stress significantly contributes to PD pathogenesis and MPTP neurotoxicity. It was found that MPP^+ could induce oxidative stress and apoptosis in SH-SY5Y cells (Cassarino *et al.*, 1997), and oxidants also contribute to the neurodegenerative process in cellular models of PD (Anantharam *et al.*, 2007). Mitochondrial dysfunction can also lead to oxidative stress (Mosley et al. 2006). The MPP^+-induced decrease in mitochondrial dehydrogenase activity may directly relate to its marked attenuation of ROS and RNS production. Thus, the effect of MPP^+ on the indicators of ROS and RNS were measured in the present study. MPP^+ did in fact induce a higher production of ROS, which was also

demonstrated in other studies from SH-SY5Y and other cells (Anantharam *et al.*, 2007; Jung *et al.*, 2007; Kim *et al.*, 2007).

Several studies have shown that plasma membrane NADPH-oxidase is the primary enzyme responsible for ROS production (reviewed by Geiszt, 2006; reviewed by Infanger *et al.*, 2006). Following protein kinase C (PKC) activation, the NADPH-oxidase $p47^{phox}$ component becomes phosphorylated, promoting its translocation to the plasma membrane along with $p67^{phox}$, $p40^{phox}$, and the small GTP-binding protein Rac. Together, they then form an active enzyme complex (reviewed by Ray and Shah, 2005). This occurs through an increased amount of assembled complexes and/or changes in the phosphorylation status of $p47^{phox}$ (Wu *et al.*, 2003).

Since $p47^{phox}$ is the first main isoform during the activation of the NADPH-oxidase phox holoenzyme, the present study, at both mRNA and protein levels, demonstrated that the NADPH-oxidase $p47^{phox}$ was significantly increased by MPP^+ treatment. This is supported by a recent study showing that NADPH-oxidase has a critical role in the oxidative damage in PD, and targeting this enzyme may thus lead to novel therapies for the disease (Anantharam *et al.*, 2007). Although this result is consistent with findings by Zhang *et al.* on mesencephalic neuron-glia cultures (Zhang *et al.*, 2008), this study, for the first time, reported that an increase in MPP^+-triggered $p47^{phox}$ was found in human neuroblastoma cells. The effects of MPP^+ on the NADPH-oxidase $p47^{phox}$ also indicate that NADPH-oxidase may be one of the main sources of increased ROS under MPP^+ insult. Another main source of the MPP^+-induced increase in ROS may be the inhibition of complex I from the ETC, which may be induced by mitochondrial dysfunction, as mentioned in Section

1.4.3.1 in Chapter 1. In addition, the production of the oxidant RNS was also increased by MPP^+, which is also consistent with Dennis and Bennett's findings in SH-SY5Y cells (Dennis and Bennett, 2003). Furthermore, MPP^+ may mediate the production and release of NO through the inhibition of complex I (Schulz *et al.*, 1997). NO reacted with O_2^- may cause cell death due to their production of $ONOO^-$. NO can be produced by nNOS, which is mainly expressed in cells of neuronal origin, as a neuromodulator of the central and peripheral nervous systems (reviewed by Bredt, 1999). In the present study, nNOS was significantly decreased to less than half of the control under MPP^+ treatment, which is consistent with the finding by Shang *et al.* (2005). However, the finding in this study seems to contradict another finding, which reported nNOS may contribute to MPP^+-induced toxicity *in vitro* (O'Byrne and Tipton, 2002). This could be explained by the fact that in the central nervous system, the NO produced by nNOS also serves as an important neurotransmitter and regulates neuronal activity (reviewed by de Col *et al.*, 2003). Therapeutic interventions with NOS inhibitors did not reduce MPP^+-induced toxicity, and in some cases, NOS inhibition was even deleterious rather than protective (MacKenzie *et al.*, 1994, Barc *et al.*, 2001; Mohanakumar *et al.*, 2002). Thus, NOS could be deleterious, depending on the source of NO and the amount produced. It is difficult to explain why MPP^+ enhanced NO production but suppressed the nNOS expression in this study. It is possible that some NO might be produced by non-enzyme-dependent pathways, which has also been reported by another group (Zweier *et al.*, 1999).

2.4.2 The effect of MPP^+ on antioxidant enzymes and antioxidants

On the other hand, for the anti-oxidative system, mRNA expressions of the antioxidants SOD, catalase and GSHpx and the level of GSH were significantly up-regulated by MPP^+. These findings are in line with other studies, which report that MPP^+ increased SOD, catalase and GSHpx activity, and increased the mRNA expressions of SOD and catalase (Cassarino *et al.*, 1997; Amazzal *et al.*, 2007). By contrast, another study reported that SOD and catalase activities were inhibited by MPP^+ (Jung *et al.*, 2007). The inconsistency may result from the various time courses of the treatments and the different cell lines used. However, there could be other explanations. MPP^+, through impairing mitochondrial function and other pathways, induces more O_2^- to be released from the cells, which are consequently converted into H_2O_2 by the action of SOD. Thus, the increased production of O_2^- requires more SOD in the cells to produce H_2O_2, which may be the mechanism underlying the increase in SOD after MPP^+ exposure. Furthermore, to detoxify the oxidants in the cells, more catalase, GSH and GSHpx are needed to remove the increased amount of H_2O_2. Thus, increased levels of catalase, GSH and GSHpx were observed.

The findings from the present study together form, for the first time, a clear picture of the interrelationship between the oxidants and the antioxidants in the cellular model of PD. MPP^+ induces mRNA expressions of pro-oxidant enzymes, and the enzymes in turn produce higher oxidants. Thus, the increase in antioxidant function may act as a feedback mechanism. Although antioxidants are increased to defend against oxidants, the fact is that decreased mitochondrial dehydrogenase activity still occurs after MPP^+ administration, which may indicate that an imbalance between oxidative and anti-oxidative systems exists.

There could be other factors involved in the decrease of mitochondrial dehydrogenase activity, such as inflammation and apoptosis.

2.4.3 The effect of EPA treatment alone on mitochondrial dehydrogenase activity, oxidant production, pro-oxidant enzymes and antioxidant enzymes

As discussed in Section 1.6.4 in Chapter 1, EPA, as an essential n-3 fatty acid, can improve cell function by increasing membrane fluidity. Adequate levels of EPA and other n-3 fatty acids in membrane systems are critical to cell survival, growth, repair and a myriad of other functions (Okuyama *et al.*, 2008; Richard *et al.*, 2008; Schmitz and Ecker, 2008). Indeed, it is found that EPA by itself caused an increase in mitochondrial dehydrogenase activity in fully differentiated SH-SY5Y cells, which is consistent with previous findings in our lab (Kou *et al.*, 2008). There could be several mechanisms by which EPA increases mitochondrial dehydrogenase activity.

First, n-3 fatty acids, including EPA, are antioxidants (Okuyama *et al.*, 2008; Richard *et al.*, 2008) and may reduce oxidant productions by alleviating mitochondrial dysfunction in an imbalanced condition between oxidative and anti-oxidative systems. However, no evidence has shown the anti-oxidative effect of pure EPA on brain cells in baseline conditions. From the results of this study, EPA did not significantly affect ROS and RNS productions by itself. At the enzymatic level, the pro-oxidant enzyme NADPH-oxidase, which is the main source of ROS, was also unchanged by EPA, which is consistent with the findings on the production of ROS. Furthermore, the results demonstrated that nNOS was suppressed by EPA. This result does not match the finding that the NO production is unaffected by EPA.

As discussed previously, this difference between changes in nNOS and NO may indicate that NO might be also be produced by non-enzyme dependent mechanisms. Nevertheless, these results demonstrated that EPA did not interfere with anti-oxidative functions.

Secondly, EPA may increase mitochondrial dehydrogenase activity by promoting antioxidant protection, since antioxidant GSH levels and antioxidant enzyme activities were significantly increased after EPA and DHA treatments (Kim and Chung, 2007). EPA was also found to increase the SOD mRNA gene expression in rat hepatocytes (Sohma *et al.*, 2007). However, few studies have evaluated the effects of EPA on antioxidants in brain cells. In the present study, EPA did not affect mRNA expressions of the antioxidant enzymes SOD, catalase and GSHpx, and it also did not affect the concentration of the antioxidant GSH. Thus, EPA treatment, under normal conditions, does not affect the normal function of oxidants and antioxidants. However, EPA itself did indeed enhance mitochondrial dehydrogenase activity, which indicates that other mechanisms might be involved. For example, our team has recently reported that EPA can up-regulate the expressions of neurotrophins and their receptors (Kou *et al.*, 2008; Song *et al.*, 2009). This could be studied in the future.

2.4.4 Whether and how EPA attenuates the effect of MPP^+

In the fully differentiated SH-SY5Y cells that received both EPA and MPP^+ treatments, EPA provided protection against the MPP^+-mediated loss of mitochondrial dehydrogenase activity. This is the first study to demonstrate that EPA protects cells against the effect of

MPP^+. Furthermore, the present study is also the first to explore the possible mechanisms by which this protection occurs.

First, the effects of EPA on the oxidative system were studied. As previously discussed, MPP^+-induced oxidative stress may suppress mitochondrial dehydrogenase activity. EPA, as an antioxidant, can attenuate oxidative stress and down-regulate the oxidant production from mitochondrial dysfunction (Kim and Chung, 2007; McMillin *et al.*, 1992). Indeed, EPA attenuated the increase in the productions of both RNS and ROS, which were induced by MPP^+ (at lower doses) in the present study.

Second, the proteins in the membrane could be more diversely interacting with the surrounding lipids through increased membrane fluidity by EPA and other n-3 fatty acids (reviewed by Kidd, 2007). In addition, many of the effects of EPA are exerted through altered gene expressions (reviewed by Schmitz and Ecker, 2008). Since EPA can modulate gene and protein expressions, the effects of EPA on the gene and/or protein expressions of pro-oxidant enzymes were investigated in the present study. The ROS enzyme, NADPH-oxidase ($p47^{phox}$), was down-regulated by EPA, as evidenced by both its mRNA and protein expressions. Thus, these results suggest that EPA may reduce the ROS production by decreasing NADPH-oxidase. However, since NO was decreased by EPA but nNOS was unaffected, it is suggested that EPA may modulate NO via another mechanism. This should be studied in the future. It is important to note here from the results of the experiments in which MPP^+ was administered in different doses and those in which EPA and MPP^+ were combined, cell sensitivity to MPP^+ varied. The level of MPP^+-induced changes in the MTT

assay and DAF-DA test are different. The reason for the induced variation is unclear; however, it may be caused by the presence of different generations of cells and cell variation to MPP^+, which may be the limitation of the model.

Third, EPA has previously been reported to exert an antioxidant effect by reducing oxidant productions (Kim and Chung, 2007; Sohma *et al.*, 2007). In the present study, EPA decreased the effect of MPP^+ on the GSHpx gene expression, but had no effect on other antioxidants and antioxidant enzymes in dopaminergic neuron-like cells. Although EPA was unable to increase other antioxidant and antioxidant enzymes, the result shows that EPA attenuated the effect of MPP^+ on the mRNA expression of GSHpx, suggesting that EPA does have some anti-oxidative functions. Due to the overall lack of EPA effects on the modulation of MPP^+-mediated antioxidant content and the antioxidant enzymes, its modulation of antioxidant enzymes SOD, GSHpx and catalase and antioxidant GSH may be not the primary pathway by which EPA attenuated the MPP^+-induced loss of mitochondrial dehydrogenase activity. Thus, the attenuating effect of EPA on the oxidants ROS and RNS, and the pro-oxidant enzyme NADPH-oxidase could be the primary pathways for protecting mitochondrial dehydrogenase activity against MPP^+.

In summary, the present study demonstrated that MPP^+ administration reduced mitochondrial dehydrogenase activity, increased oxidant productions and up-regulated the mRNA expression of the pro-oxidant enzyme NADPH-oxidase. The MPP^+-induced oxidative stress may activate the antioxidants SOD, GSHpx and catalase. EPA, in normal conditions, enhanced mitochondrial dehydrogenase activity, which was not associated with

its effect on oxidative and anti-oxidative systems, suggesting that other protective mechanisms are involved. In the PD model, EPA attenuated the effect of MPP^+ on mitochondrial dehydrogenase activity by reducing the production of oxidants and the pro-oxidant enzyme NADPH-oxidase, in turn preventing an imbalance between oxidative and anti-oxidative systems.

Chapter 3

Efficacy of EPA against MPTP Effects on Oxidative Stress and the Fatty Acid Profile in Brain Slice Model and Chronic C57BL/6 Mouse Model

3.1 Introduction

PD results from the loss of dopaminergic neurons in the SN and the striatum, a process to which oxidative stress has been shown to contribute significantly (reviewed by Jenner *et al.*, 1992). Oxidative stress occurs when the generation of oxidants in a system exceeds the system's ability to detoxify or eliminate (Mena *et al.*, 2008). Major oxidants in mammalian cells include ROS and RNS, triggered by NADPH-oxidase and NOS respectively. The most common antioxidant and antioxidant enzymes are GSH, SOD, GSHpx and catalase (reviewed by Özben, 1998). Their functions have already been introduced in Section 1.4.2 of Chapter 1.

To study the oxidative stress mechanism of PD and evaluate new treatments, cellular and animal models have been used. As introduced in Section 1.5.3, Chapter 1, MPTP and its metabolite MPP^+ were applied to induce PD models because MPTP could selectively damage dopaminergic neurons in the SNpc and impair motor function (reviewed by Burns, 1984). MPTP-induced oxidant production may be responsible for these changes (Mizuno *et al.*, 2008). In Chapter 2, it was demonstrated that the cellular mechanism of oxidative stress could be induced by MPP^+ in dopaminergic neuron-like cells. Compared to the *in vitro* model, an *in vivo* model can allow us to: 1) manipulate acute and chronic PD procedures; 2) study the neuropathological pathways specifically in the area of PD neurodegeneration and 3) evaluate the relationship between oxidative stress and PD symptomatology. Therefore, the present chapter aims to further understand the role of oxidative stress in PD at systematical level by using two animal models. One is the MPP^+-treated mouse brain slice

model (acute MPTP model), and the other one is the MPTP/probenecid injection mouse model (chronic MPTP model).

Studies with brain slices, although not as widely used as *in vivo* models, are getting more attention from many researchers. In the etiology of PD, environmental contaminants have been considered as an essential factor. Environmental toxins can be chronic or acute, such as those caused by the acute toxicity of dieldrin, a possible environmental risk factor of PD (Kitazawa *et al.*, 2001). Brain slices have been used for studying the mechanism of the acute insult of a neurotoxin (direct effect), and rodent striatal slices have been used as a model to study the direct and acute effects of MPP^+ neurotoxicity. After incubating with MPP^+, striatal slices can be used as an acute PD model or as a model to explore the early stage of PD neurodegeneration. Evidence has shown that 1) oxidative stress is generated following striatal slice exposure to MPP^+ (Ambrosio *et al.*, 1996; Sriram *et al.*, 1997; Gille *et al.*, 2004) and 2) MPP^+ incubation with brain slices for a short time can induce lipid peroxidation (Ambrosio *et al.*, 1996). Pro-oxidant and antioxidant enzymes can control the production and detoxification of oxidants, respectively. Thus, they may play an important role in the imbalance between oxidants and antioxidants, which may contribute to the neurodegeneration in PD. However, the role of the pro-oxidant enzyme NADPH-oxidase in MPP^+-induced oxidation, and the role of antioxidant enzymes GSHpx and catalase in striatal slices remain unknown in MPTP models. Thus, in this chapter, the first aim is to discuss the mechanism of oxidative stress in the MPP^+-induced acute PD model, using striatal slices.

As mentioned above, brain slice experiments can reveal the direct and acute effects of MPTP in the oxidative and anti-oxidative systems in the brain. Since PD is a chronic and progressive disease, the changes in oxidative stress in brain slices cannot reflect the changes in the late stage. Thus, in the present study, the chronic MPTP/probenecid-induced PD model has been used to better understand oxidative stress in the late stage of PD, including oxidative and anti-oxidative abnormalities. In the first step of this study, the pro-oxidant enzymes were studied as a reflection of the changes in the oxidative system. Wu *et al.* (2003) have recently demonstrated that NADPH oxidase-deficient mice are less sensitive to MPTP, which indicates that this pro-oxidant enzyme is important in MPTP-induced oxidative stress. Other pro-oxidant enzymes may also contribute to oxidative stress in MPTP models. For example, a deficiency of iNOS was found to suppress the toxicity of MPTP (Liberatore *et al.*, 1999; Gille *et al.*, 2004). Secondly, the antioxidant enzymes were studied as the biomarkers of changes in the anti-oxidative system. Previously, MPTP treatment was reported to significantly decrease GSHpx activity in both the SN and the striatum, without affecting other antioxidants (Genc *et al.*, 2002). In another experiment, the activity of GSHpx, catalase and SOD, and the mRNA expression of GSHpx were all decreased in the striatum in mice treated with MPTP (Chen *et al.*, 2007). Still other studies reported that MPTP increased the specific activity of SOD, catalase and lipid peroxidation in both the SN and midbrain of mice (Rajeswari, 2006). These inconsistent results indicate that more work in this area is needed before the contribution of oxidative stress to PD pathology is understood. Thus, the chronic MPTP/probenecid-induced PD model was used to clarify the conflicting results from other PD models. Therefore, the second aim of this

study was to investigate the chronic effects of MPTP on pro-oxidant enzymes and antioxidant enzymes as mentioned above, in the chronic MPTP/probenecid model.

The brain slice and chronic MPTP/probenecid models can be used not only to better understand the mechanism of PD neuropathology, such as oxidative and anti-oxidative systems in PD, but it also to test and develop new therapies for PD. Oxidative stress can cause lipid peroxidation of PUFA (reviewed by Rokyta *et al.*, 1996). As the consequence, membrane concentrations of PUFA, such as the n-3 fatty acids α-LA, EPA, DPA and DHA, and the n-6 fatty acids LA, GLA and AA, may be reduced. These PUFA have important roles in supporting brain functions, and therefore a shortage of these PUFA may cause a higher risk of brain pathology, such as for the development of PD (Logroscino *et al.*, 1996; Anderson *et al.*, 1999; Johnson *et al.*, 1999; Chen *et al.*, 2003; de Lau *et al.*, 2005). The intake of n-3 fatty acids could change the components of n-3 and n-6 fatty acids in the mitochondrial and cell membrane of the brain, which, in turn, can regulate multiple brain functions (Bousquet *et al.*, 2008). Bousquet and co-workers have recently reported that a high n-3 PUFA dietary intake provides neuroprotective actions by maintaining DA levels in the striatal tissues of an MPTP mouse model (Bousquet *et al.*, 2008). Conversely, n-3 fatty acid deficiency in mouse models was reported to reduce antioxidants in the brain, cause microglial activation and increase oxidative stress, all of which can increase the risk of certain DA-associated neurological disorders (Kuperstein *et al.*, 2008; Lavialle *et al.*, 2008; McNamara *et al.*, 2008). Evidence from an *in vivo* model of pancreatic cancer showed that the pro-oxidant enzyme iNOS was decreased after n-3 fatty acid intake (Swamy *et al.*, 2008). Of particular interest to the present study, EPA, as one of n-3 fatty acids, has been

demonstrated to reduce oxidative stress and inflammation, and protect neurons (Song *et al.*, 2004), suggesting the potential to prevent or treat PD. Although there is plenty of evidence showing that EPA could enhance brain function and prevent oxidative stress, there has been little investigation on the effect of EPA in PD models or patients. Thus, the third aim of this study was to evaluate the capability of EPA to attenuate oxidative stress in two animal studies using the brain slice and the chronic models. The final aim of this study was to clarify the interrelation between MPTP/MPP^+-induced oxidative stress and changes in n-3 and n-6 fatty acids with and without EPA treatments, in the two animal models.

Based on these four aims, the hypotheses were: 1) MPTP would induce oxidative stress by increasing pro-oxidant enzymes, decreasing antioxidant enzymes, and changing the PUFA composition in the brain slice model and the chronic model; 2) EPA may attenuate the effect of MPTP on oxidative stress in these two models and 3) MPTP/MPP^+ may induce oxidative stress and changes in n-3 and n-6 fatty acids, and EPA may attenuate them.

To test these hypotheses, in the first PD model (brain slice experiment), C57BL/6 mice were pre-treated with a diet enriched with ethyl-EPA or a control diet (palm oil) for 6 weeks. After decapitation, mouse brains were removed and sliced for MPP^+ incubation. Following incubation with MPP^+, the fatty acid profile in the midbrain and frontal cortex, which are related to basal ganglia functions, was analyzed by GC. The mRNA expression of the pro-oxidant enzyme NADPH-oxidase and mRNA expressions of the antioxidant enzymes GSHpx and catalase in the striatal slices were measured by quantitative PCR. For the chronic mouse model, C57BL/6 mice were fed with an ethyl-EPA diet for a total of 12

weeks. After feeding for 6 weeks, they were administered a chronic MPTP/probenecid regiment for one month. Animal motor behaviors were tested by co-workers following two-week of MPTP treatments (data not shown). The mice were sacrificed after the behavioral test. To test the changes of fatty acids in the brain, the fatty acid profile in the frontal cortex, related to basal ganglia functions, was analyzed by GC. The mRNA expressions of the pro-oxidant enzymes NADPH-oxidase and iNOS, the mRNA expressions of the antioxidant enzymes SOD, GSHpx and catalase in the midbrain and striatum were measured by quantitative PCR.

3.2 Method

3.2.1 Animals: All experimental procedures were performed according to the guidelines of the *Canadian Council on Animal Care (CCAC)* at the University of Prince Edward Island. Male C57BL/6 mice (Charles River, Canada), 10 weeks of age and weighing 25g, were acclimated for 1 week in the animal colony. The mice were individually caged under standard conditions throughout the experiments, with free access to food and water and maintained on a 12 hour light-dark cycle with the room temperature set at 22±1°C.

3.2.2 Diet: Following acclimation, 20 mice in the brain slice study and 40 mice in the chronic MPTP/probenecid study were fed a diet consisting of normal chow (PMI® Richmond, Indiana, US) mixed with 0.8% ethyl-EPA (Amarin Neuroscience Ltd, UK) (brain slice study: n = 10; MPTP/probenecid: n = 20) or 0.8% palm oil (Sigma, Canada) (brain slice study: n = 10; MPTP/probenecid: n = 20). Palm oil was used as the control diet,

since it ensures comparable caloric values, with very low amounts of LA (C18:2n6) and no n-3 fatty acids (Song *et al.*, 2008). For the brain slice experiment, mice were fed for 6 weeks before being sacrificed. For the MPTP-induced chronic model, mice were fed their designated diet for 6 weeks before MPTP injections. During and after MPTP injections, animals were maintained on the same diet. The total feeding time for animals in the chronic study was 12 weeks. Food was freshly prepared every three days, fed daily and stored in -20°C overnight.

3.2.3 Drug treatments

3.2.3.1 Brain slice

MPP^+ iodide (Sigma, Canada) was dissolved in sterile deuterium-depleted water (dd H_2O) to generate a stock concentration of 10mM. After aliquoting, MPP^+ was frozen at -80°C. The dose of 20 μM MPP^+ has been tested at our lab and revealed that it can induce the most depletion of DA.

3.2.3.2 Chronic MPTP/probenecid

100 mg MPTP hydrochloride (Sigma, Canada) was dissolved in 20 ml sterile saline, and diluted to a 5mg/ml solution for injections. 400 mg of probenecid (Sigma, Canada) was dissolved in 16 ml 0.1N NaOH, which was adjusted with 1M Tris-HCl (pH 6.8) to a pH of 7.4, and the final volume was adjusted to 32 ml with dd H_2O. Solutions were prepared freshly for each injection.

3.2.4 Animal sacrifice and brain slice preparation

Following the 6 week feeding trial, the animals were anesthetized with halothane and decapitated. Their brains were rapidly removed and placed in ice cold, oxygenated bicarbonate-buffered (95% O_2:5% CO_2) artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose. A 0.25cm^2 block of brain tissue containing the striatum was glued to a cutting surface and hemisected. Coronal (200-400μM) slices were prepared using a Leica VT1000S microtome (Leica Microsystems). Following sectioning, the slices were left to equilibrate in ACSF for approximately 1 hour in a recovery chamber. The slices were then transferred to 6-well plates containing oxygenated bicarbonate-buffered ACSF with or without MPP^+. Of each animal brain, the slices of one hemisection were incubated for 4 hours in ACSF with 20 μM MPP^+ and the slices of the other hemisection were incubated in ACSF without MPP^+ (as control condition). ACSF was held at room temperature (21±1°C). This model contained 4 different treatment groups, including palm oil with ACSF medium (n = 10); palm oil with MPP^+-treated ACSF medium (n = 10); EPA with ACSF medium (n = 10) and EPA with MPP^+-treated ACSF medium (n = 10). Four hours after incubation, the brain slices containing the striatum were placed on the glass slide under a microscope. The striatum, midbrain and frontal cortex were dissected according to mouse atlas from http://mouse.brain-map.org and then stored in the -80°C freezer for further studies.

The brain region identified as the frontal cortex (containing motor area 1 and 2) in this work corresponds to the dissection of the cortical area in each 300-400 micron slice between Bregma coordinates from 0 to 2.0 mm with lateral coordinates from 0.2-1.5 mm.

The coordinates for the striatum were the same as those of the frontal cortex, with the lateral coordinates at 1-3 mm, plus, the core region of the nucleus accumbens and the corpus callosum inside the lateral ventricles. The midbrain was dissected between Bregma coordinates -3 and -5 (According to http://mouse.brain-map.org).

3.2.5 MPTP/ probenecid administration-induced chronic PD model

After being fed with a control or EPA diet for 6 weeks, animals were administered the MPTP/probenecid injections. Five days before the first injection, animals treated with MPTP were placed in a fume hood for safety purposes. The temperature of the injection room was kept at 24°C to prevent hypothermia (reviewed by Przedborski and Vila, 2003). MPTP at a concentration of 25 mg/kg combined with probenecid, or saline combined with probenecid (250 mg/kg), was injected 10 times at 3.5 days intervals. MPTP or saline was subcutaneously administered and probenecid was intraperitoneally administered. Probenecid was chosen to suppress the renal excretion of MPTP and its metabolite MPP^+, thereby maintaining the MPTP effects for the full 3.5 days injection interval (Lau *et al.*, 1990; Petroske *et al.*, 2001). This model contained four different treatment groups (total n = 38, two mice were dead after injection): palm oil with saline/probenecid (n = 9); palm oil with MPTP/probenecid injection (n = 11); EPA with saline/probenecid injection (n = 10), and EPA with MPTP/probenecid injection (n = 8). Upon completion of the MPTP/probenecid administrations, the mice were sacrificed by cervical dislocation. Brains were rapidly removed, and the striatum, frontal cortexes and midbrains were dissected on ice according to mouse atlas from http://mouse.brain-map.org. The frontal cortex was obtained by removing the olfactory bulb and making a cut 1mm caudal to the beginning of

the brain proper. The striatum was obtained by making a second cut 1.2 mm caudal to the first cut. The midbrain region was removed under the cerebellum by cutting the anterior edge of the cortex and the posterior edge of the brain stem. The brain samples were stored at -80°C.

3.2.6 Fatty acid extraction

The frontal cortex and midbrain tissues from the brain slices of the PD model and the frontal cortex tissues from the chronic PD model were collected for fatty acid extraction. The Folch method was used to extract total lipids (Folch *et al.*, 1957). C13:0 was added as an internal standard before extraction. Brain tissues were homogenized with 5 ml chloroform/methanol solution (2:1). To rinse the polytron, another 2.5 ml chloroform/methanol solution was added to the homogenate. After dispersion, the entire mixture was agitated for 60 minutes in an orbital shaker at room temperature. Two ml of 1% NaCl was then added to the brain tissue homogenate. After vortexing, the mixture was centrifuged at 2990 xg to separate the two phases. After the centrifugating and siphoning of the upper phase, the lower lipid-containing chloroform phase was dried under nitrogen stream. The dried fatty acid samples were incubated in a water bath at 80°C with 5 ml of ethanolic KOH (0.4 M). After cooling, 2.5 ml of 1% NaCl and 1.5 ml of hexane were added. After vortexing and phase separation, the top organic layer was discarded. Another 1.5 ml of hexane was added, and the vortexing and separation were repeated. HCl solution (35%) was added to adjust the pH to 2-2.5, followed by 2ml hexane. Following vortexing and phase separation, the top layer was dried under nitrogen stream. 1.5 ml of 3 M methanolic HCl was added in to the dried fatty acids and incubated in a water bath at 95°C for 60

minutes. After cooling, 2.5 ml of 1% NaCl and 1.5 ml hexane was added to the samples. After vortexing and phase separation, the top layer containing fatty acid methyl esters (FAME), which contained both free and bound fatty acids, was collected for GC analysis.

3.2.7 Fatty acid analyses by GC

FAME were analyzed by GC using a Hewlett Packard 6890 (Palo Alto, CA) equipped with a flame ionization detector. The FAME were separated on a 60 m Agilent 122-2361 DB-23 capillary column (0.25 mm diameter, 0.15 μm coating thickness; Restek, Bellefonte, PA), using helium at a flow rate of 2.1 ml/minutes with split ratios of 48:1 and 20:1. The chromatographic parameters included an oven starting temperature of 130°C, which was increased at 6°C/minute to 225°C, where it was held for 20 minutes before being increased to 250°C at 15°C/minutes, with a final holding duration of 5 minutes. The injector and detector temperatures were held constant at 220°C and 230°C, respectively. Peaks were identified by comparing retention times with external FAME standard mixtures and PUFA standards from Sigma and Nu-Chek Prep. Fatty acid profile was expressed as a percentage of the total microgram of the tissue (weight percentage). If one fatty acid could not be detected or separated from the other fatty acids, it was excluded.

3.2.8 RNA extraction

The striatal slices from the brain slice study and the mouse midbrains and striatum from the chronic mouse study were utilized for RNA extraction. The RNA extraction method followed was as described by the TRI-reagent technical bulletin. The details of this method were presented in Chapter 2, Section 2.2.7.

3.2.9 cDNA synthesis

QuantiTect Reverse Transcription (Qiangen, 205311) was carried out to synthesize cDNA from the collected RNA. For all the procedures, see Section 2.2.8 in Chapter 2.

3.2.10 Quantitative PCR

A QuantiTect SYBR Green PCR Kit was used for quantitative PCR. All PCR reactions were performed on Rotor Gene 6000 Quantitative PCR Machine. For the details of the PCR, see Section 2.2.9 in Chapter 2. The specific forward/reverse primers used were: GSHpx1: 5′- TTTGAGAAGTTCCTGGTGGG-3′/5′- TGCAGCCAGTAATCACCAAG-3′; SOD2: 5′- TGTGTACCTTGTCAGGGA-3′/5′- ACCTTTCTCAGTAGCGG-3′; Catalase: 5′- CCTCGTTCAGGATGTGGTTT-3′/5′- TCTGGTGATATCGTGGGTGA-3′; iNOS: 5′- CACCTTGGAGTTCACCCAGT-3′/5′- ACCACTCGTACTTGGGATGC-3′; $P47^{phox}$: 5′- CGAGAAGAGTTCGGGAACAG-3′/5′- TCCAGATAGGATGCAGGGAC-3′; and β-actin reference: 5′- TGTTACCAACTGGGACGACA-3′/5′- CTGGGTCATCTTTTCACGGT-3′. The reaction included 55 cycles. After 55 cycles, a melting curve was generated to determine primer specificity and identity, and each melting curve of the primer is presented in an appendix. The ΔΔCT method (Livak and Schmittgen, 2001) was used to normalize gene expression data; see Section 2.2.9 in Chapter 2.

Due to the low amount of mouse brain tissues in the brain slices, and with only half of the brain area used in chronic PD models (half of each brain was used for an alternate experiment, not included in this thesis), mRNA extraction failed for some tissues. In the

brain slice model, 26 mRNA samples were successfully extracted from the slice tissues (Control: n = 6; EPA alone: n = 6; MPP^+: n = 6; EPA and MPP^+ combined group: n = 8). In the midbrain of the chronic MPTP model, 30 mRNA samples were extracted (Control: n = 7; EPA alone: n = 8; MPP^+: n = 7; EPA and MPP^+ combined group: n = 8). In the striatum of the chronic MPTP model, 29 mRNA samples were extracted (Control: n = 8; EPA alone: n = 8; MPP^+: n = 6; EPA and MPP^+ combined group: n = 7). However, the mRNA amounts extracted from mouse brain tissues were very low, and may have been insufficient to detect the target genes. The sample size was also variable due to this factor.

3.2.11 Statistical analyses

Data were analyzed using GraphPad Prism 4.0 and presented as the mean ± SEM. As two factors were investigated in this experiment, the diet factor and the MPTP/MPP^+ treatment factor, a two-way ANOVA with the *post-hoc* Bonferroni test was applied to analyze the results. The *post-hoc* Bonferroni test indicated that the results were compared at different levels, and three levels of interest are presented and discussed, including: the results of the MPP^+ group were compared with that of the control group; the results of the EPA treatment group was compared with results of the control diet group; and the EPA and MPTP/MPP^+ combination group was compared with the group treated with MPTP/MPP^+ alone. P values less than 0.05 were considered to be statistically significant. Extreme values checked by box plot test were removed from experiment. The number of removed outliers is shown in the result section. If no outlier is shown, it indicates that no outlier was removed from the statistical analyses. The data without signals in both GC and PCR were removed before the statistical analyses. The number of replicates in each test is presented in the figure text.

3.3 Results

3.3.1 Fatty acid analyses in the brain slice model

To analyze the fatty acid profile in brain slices of the PD model, GC was used. The experiment contained four groups: the control group (previously fed a palm oil-enriched diet and then cultured with control ACSF medium), the group treated with EPA alone (previously fed an EPA-enriched diet and then cultured with control ACSF medium), the group treated with MPP^+ alone (previously fed a palm oil-enriched diet and then cultured with ACSF medium containing MPP^+), and the group treated with both EPA and MPP^+ (previously fed an EPA-enriched diet and then cultured with ACSF medium containing MPP^+).

In this study, the two-way ANOVA revealed that the MPP^+ ($F1, 35 = 0.4967$, $p = 0.4856$) and diet factors ($F1, 35 = 1.508$, $p = 0.2276$) both had no effects on the concentration of n-3 fatty acid α-LA in brain slice tissues. However, there was an interaction between MPP^+ and diet ($F1, 35 = 5.429$, $p = 0.0257$) (Figure 3.1.1A), which indicates that MPP^+ effects depend on the diet.

With regard to EPA concentrations in the brain slice tissues, the two-way ANOVA revealed that the MPP^+ factor did not have a main effect on n-3 fatty acid EPA concentrations ($F1,33 = 0.0071$, $p = 0.9338$), while the diet factor significantly affected EPA concentrations in the brain slice tissues ($F1, 33 = 98.69$, $p<0.0001$). There was no interaction between MPP^+ and diet ($F1, 33 = 0.3632$, $p = 0.5508$). The *post-hoc* test showed that EPA concentrations were

significantly increased by the EPA treatment alone when compared to the control group ($t = 7.548$, $p<0.001$). In the group treated with both EPA and MPP^+, EPA concentrations increased when compared to the MPP^+ alone group ($t = 6.515$, $p<0.001$) (Figure 3.1.1B).

In addition, the two-way ANOVA indicated that the MPP^+ factor was not associated with a significant change in the concentration of the n-3 fatty acid DPA ($F1, 34 = 0.1883$, $p<0.6671$) (one outlier was removed from MPP^+ alone group), while the diet factor was associated with a significant change in DPA concentrations in brain slice tissues ($F1, 34 = 28.90$, $p<0.0001$). There was no interaction between MPP^+ and diet ($F1, 34 = 0.1619$, $p = 0.6899$). The *post-hoc* test showed that DPA concentrations were significantly increased by EPA treatment alone when compared to the control treatment ($t = 3.719$, $p<0.01$). DPA concentrations in the brain slice tissues of the group treated with a combination of EPA and MPP^+ was also significantly increased when compared to the group treated with MPP^+ alone ($t = 3.855$, $p<0.001$) (Figure 3.1.1C).

Furthermore, neither MPP^+ ($F1, 32 = 1.175$, $p = 0.1104$) nor EPA ($F1, 32 = 0.3443$, $p = 0.2865$) had any effects on DHA concentrations in brain slice tissues. There was no interaction between EPA and MPP^+ ($F1, 32 = 2.696$, $p = 0.5615$) (Figure 3.1.1D).

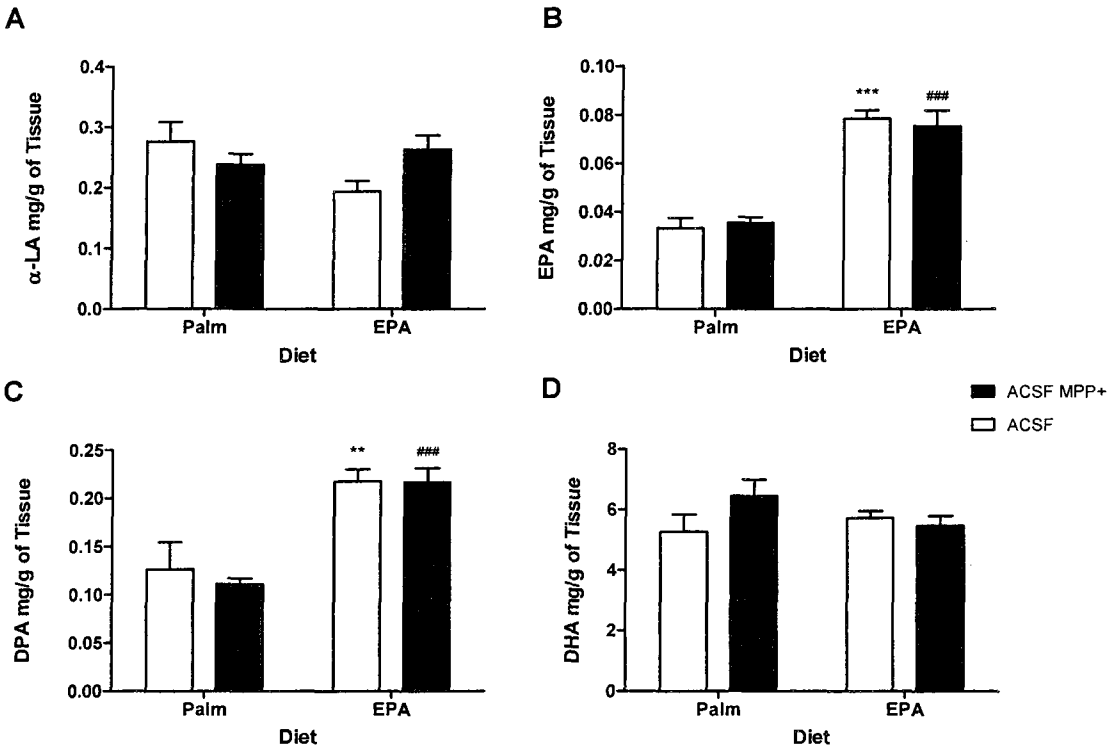

Figure 3.1.1. The effect of MPP^+ and EPA on concentrations of n-3 fatty acids in the brain slice model: GC measurement of brain (frontal cortex and midbrain) (A) α-LA (C: n = 10; E: n = 10; M: n = 9; EM: n = 10), (B) EPA (C: n = 9; E: n = 9; M: n = 10; EM: n = 9), (C) DPA (C: n = 10; E: n = 10; M: n = 8; EM: n = 10) and (D) DHA concentrations (mg/g brain tissue) (C: n = 9; E: n = 9; M: n = 9; EM: n = 9), following MPP^+ administration with 0.08% ethyl EPA or palm oil control diets. Results are expressed as mean ± SEM. *** $p<0.001$ as compared to the control, ### $p<0.001$ as compared to the MPP^+ alone group. C, Palm oil diet and ACSF; E, EPA diet and ACSF; M, Palm oil diet and ACSF with MPP^+, and EM, EPA diet and ACSF with MPP^+.

For the n-6 fatty acids, the two-way ANOVA indicated that the MPP^+ factor significantly affected the concentrations of the n-6 fatty acid LA in the brain slice tissues ($F1, 31 = 5.308$,

$p = 0.0281$), and the diet factor also had a significant effect on LA concentrations ($F1, 31 = 7.623$, $p = 0.0096$). There was no interaction between MPP^+ and diet ($F1, 31 = 6.8997$, $p = 0.3502$). The *post-hoc* test showed that the concentration of LA was significantly increased by MPP^+ when compared to the control group ($t = 2.523$, $p<0.05$). EPA treatment alone had no effect on LA concentrations when compared to the control group, but EPA significantly attenuated the MPP^+-induced increase in LA concentrations in the group with both EPA and MPP^+ treatments when compared to the MPP^+ alone group ($t = 2.457$, $p<0.05$) (Figure 3.1.2A).

Additionally, the two-way ANOVA indicated that the MPP^+ factor marginally affected the concentration of the n-6 fatty acid AA in the brain slice tissues ($F1, 32 = 4.109$, $p = 0.0511$), while the diet factor had no effect on AA concentrations ($F1, 32 = 0.5409$, $p = 0.4674$), and there was an interaction between MPP^+ and diet ($F1, 32 = 6.81$, $p = 0.0137$). The *post-hoc* test showed that the concentration of AA was significantly increased by MPP^+ ($t = 3.279$, $p<0.01$) when compared to the control group. EPA treatment alone had no effect on AA concentrations when compared to the control. However, EPA did manage to attenuate the MPP^+-induced increase in AA concentration in the group with both EPA and MPP^+ treatments when compared to the MPP^+ alone group ($t = 2.366$, $p<0.05$) (Figure 3.1.2B).

Despite clear results with AA, the GC could not detect the n-6 fatty acid GLA in the brain slice tissues due to the small amount of tissues in the sample.

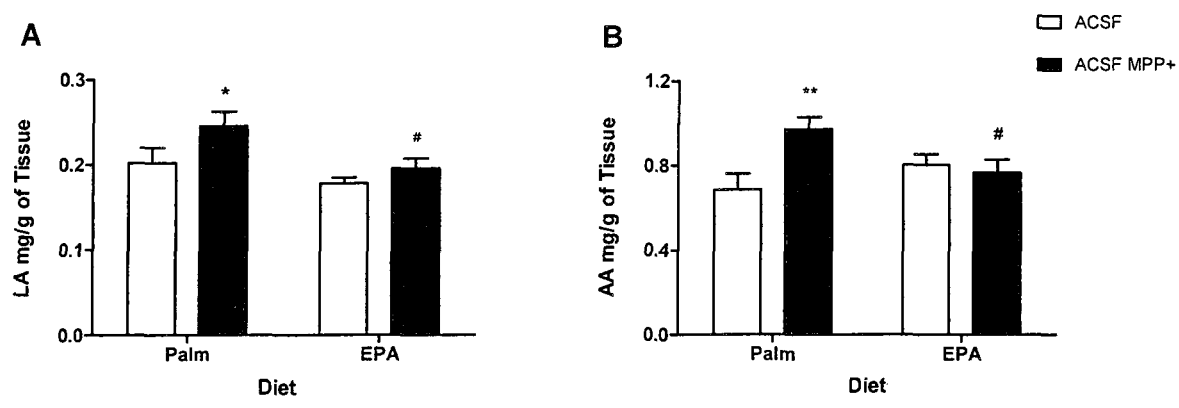

Figure 3.1.2. The effect of MPP^+ and EPA on concentrations of the n-6 fatty acids in the brain slice model: GC measurement of brain (frontal cortex and midbrain) (A) LA (C: n = 9; E: n =10; M: n = 8; EM: n = 9) and (B) AA concentrations (mg/g brain tissue) (C: n = 9; E: n = 9; M: n = 9; EM: n = 9), following MPP^+ administration with 0.08% ethyl EPA, or palm oil control diets. Effects of treatment are expressed as mean ± SEM. * $p<0.05$ as compared to the control, ** $p<0.01$ as compared to the control, # $p<0.05$ as compared to the MPP^+ alone group. C, Palm oil diet and ACSF; E, EPA diet and ACSF; M, Palm oil diet and ACSF with MPP^+, and EM, EPA diet and ACSF with MPP^+.

3.3.2 Fatty acid analysis in the chronic MPTP mouse model

To analyze the fatty acid profile in the mouse frontal cortex of the chronic PD model, GC was also applied. The experiment contained 4 groups: the control group (saline-injected animals with a palm oil diet), the group treated with MPTP (MPTP-injected animals with the palm oil diet), and the group treated with EPA alone (saline injection with an EPA diet), as well as the group treated with both EPA and MPTP (MPTP injected animals with the EPA diet). First, the GC analysis on the n-3 fatty acids revealed that neither the MPTP factor ($F1, 31 = 1.343$, $p = 0.2553$) nor the diet factor ($F1, 31 = 2.802$, $p = 0.1042$) had an effect on the concentrations of the n-3 fatty acid α-LA in mouse brain tissues (by two-way ANOVA). There was also no interaction between MPTP and diet ($F1, 31 = 0.05078$, $p = 0.8232$) (Figure 3.2.1A).

Similar to the results observed in brain slice experiment, the two-way ANOVA indicated that the MPTP factor did not affect EPA concentration in the mouse brain tissue ($F1, 32 = 3.585$, $p = 0.0674$), while the diet factor did significantly affect EPA concentration ($F1, 32 = 166.6$, $p<0.001$), and there was no interaction between MPTP and diet ($F1, 32 = 0.2377$, $p = 0.6292$). The *post-hoc* test showed that the EPA concentration was significantly increased by EPA treatment alone when compared to the control treatment ($t = 8.593$, $p<0.0001$). Feeding animals with the EPA-enriched diet also significantly increased the brain EPA concentration in the group treated with both EPA and MPTP when compared to the group treated with MPTP alone ($t = 9.691$, $p<0.001$) (Figure 3.2.1B).

With respect to the n-3 fatty acid DPA, the two-way ANOVA showed that MPTP did not affect the DPA concentration in the mouse brain tissue ($F1, 32 = 0.01492$, $p = 0.9036$), while EPA did have a significant effect ($F1, 32 = 126.3$, $p<0.001$). There was no interaction between MPTP and diet ($F1, 32 = 0.8684$, $p = 0.3584$). The *post-hoc* test showed that the DPA concentration was significantly increased by EPA treatment alone when compared to the control group ($t = 8.607$, $p<0.001$). EPA also significantly increased the DPA concentration in the brain tissues of mice treated with both EPA and MPP^+ when compared to the group treated with MPTP alone ($t = 7.289$, $p<0.001$) (Figure 3.2.1C).

Additionally, the two-way ANOVA showed that neither the MPTP factor ($F1, 31 = 1.317$, $p = 0.2599$) nor the diet factor ($F1, 31 = 2.353$, $p = 0.1352$) had an effect on the DHA concentration in mouse brain tissue. Nor was there any interaction between MPTP and diet ($F1, 31 = 0.06269$, $p = 0.8039$) (Figure 3.2.1D).

For n-6 fatty acids as well, the two-way ANOVA demonstrated that neither the MPTP factor ($F1, 30 = 1.704$, $p = 0.2017$) nor the diet factor ($F1, 30 = 0.2793$, $p = 0.611$) had any effect on LA concentrations in mouse brain tissues. There was no interaction between MPTP and diet ($F1, 30 = 2.274$, $p = 0.1420$ (Figure 3.2.2A). However, the two-way ANOVA indicated that the MPTP factor significantly affected the GLA concentration in mouse brain tissues ($F1, 31 = 8.309$, $p = 0.0071$), while the diet factor did not significantly change the concentration of this fatty acid in mouse brain tissues ($F1, 31 = 0.6886$, $p = 0.4130$) (One outlier was removed from MPTP with EPA diet group). There was also no interaction between these two factors. The *post-hoc* test showed that

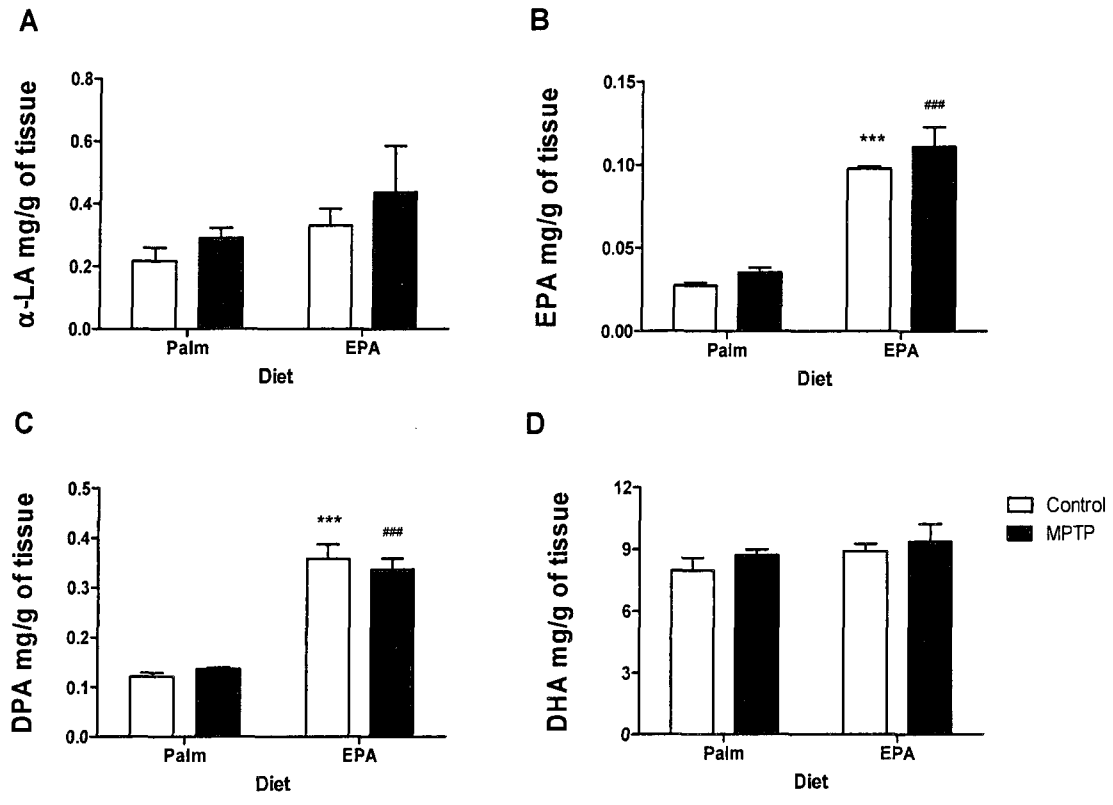

Figure 3.2.1. The effects of MPTP and EPA on concentrations of the n-3 fatty acids in the chronic model:

GC measurement of brain (frontal cortex) (A) α-LA (C: n = 8; E: n = 9; M: n = 10; EM: n = 8), (B) EPA (C: n = 8; E: n = 9; M: n = 11; EM: n = 8), (C) DPA (C: n = 8; E: n = 10; M: n = 10; EM: n = 8) and (D) DHA (C: n = 8; E: n = 9; M: n = 10; EM: n = 8) (mg/g brain tissue), following MPTP administration with 0.8% ethyl EPA or palm oil control diets. Effects of the treatments are expressed as mean ± SEM. *** $p<0.001$ as compared to the control, ### $p<0.001$ as compared to the MPTP alone group. C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

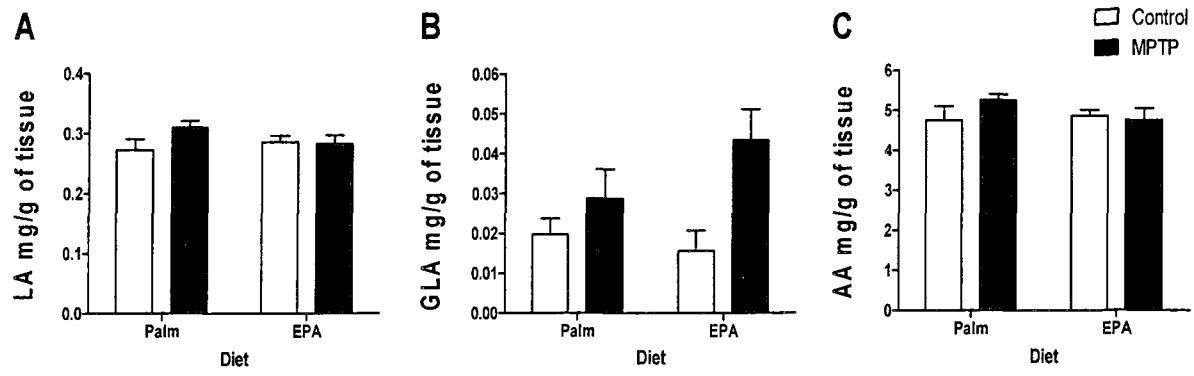

Figure 3.2.2. The effect of MPTP and EPA on concentrations of the n-6 fatty acids in the chronic model: GC measurement of brain (frontal cortex (A) LA (C: n = 8; E: n = 9; M: n = 10; EM: n = 7), (B) GLA (C: n = 9; E: n = 9; M: n = 11; EM: n = 6) and (C) AA concentrations (C: n = 8; E: n = 9; M: n = 10; EM: n = 7) (mg/g brain tissue), following MPTP administration with 0.8% ethyl EPA or palm oil diets. Effects of treatment are expressed as mean ± SEM. C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

MPTP alone did not change the GLA concentration significantly when compared to the control group (F1, 31 = 2.145, p = 0.1531) (Figure 3.2.2B). In addition, the two-way ANOVA showed that neither the MPTP factor (F1, 30 = 0.7388, p = 0.3969) nor the diet factor (F1, 30 = 0.7197, p = 0.4030) had any effect on n-6 fatty acid AA concentrations in mouse brain tissues, with also no interaction between MPTP and diet (F1, 30 = 1.643, p = 0.2098) (Figure 3.2.2C).

3.3.3 mRNA expressions of pro-oxidant and antioxidant enzymes in the striatal slice of the PD model

To test the interrelationship between pro-oxidant and antioxidant enzymes in the striatum in the early or acute stage of PD, quantitative PCR was applied to detect mRNA expressions of NADPH-oxidase, catalase and GSHpx. This experiment contained four treated groups, which were the same as those described in the Section 3.3.1 for fatty acid analyses in the brain slice model.

In the mouse striatal slices, the two-way ANOVA indicated that neither the MPP^+ factor nor the diet factor had a significant effect on mRNA expressions of catalase (MPP^+: F1, 22 = 0.1262, p = 0.7258; diet: F1, 22 = 0.06768, p = 0.7972), GSHpx (MPP^+: F1, 20 = 0.4969, p = 0.4890; diet: F1, 20 = 0.01865, p = 0.8927) (One outlier in GSHpx gene expression test was removed from MPP^+ with EPA combined group), and the NADPH-oxidase $P47^{phox}$ (MPP^+: F1, 22 = 0.006093, p = 0.9385; diet: F1, 22 = 5.532, p = 0.0280). Neither was there a significant interaction between the MPP^+ and diet factors in mRNA expressions of the

NADPH-oxidase $P47^{phox}$ ($F1, 22 = 0.2158$, $p = 0.6468$), catalase ($F1, 22 = 0.2968$, $p = 0.5914$) and GSHpx ($F1, 20 = 2.664$, $p = 0.1183$) (Figure 3.3.1 and Figure 3.3.2)

3.3.4 mRNA expressions of pro-oxidant and antioxidant enzymes in the midbrain of the chronic PD model

To test the interrelation between pro-oxidant enzymes and antioxidant enzymes in the mouse midbrain of chronic PD model, quantitative PCR was carried out. This experiment contained four groups, which were the same as those for the fatty acid analyses in the chronic model described in the Section 3.3.2.

In the midbrain of the chronic model, the two-way ANOVA showed that the MPTP factor significantly affected the mRNA expression of the pro-oxidant enzyme NADPH-oxidase ($F1, 21 = 6.56$, $p = 0.0182$), while the diet factor had no effect on the mRNA expressions of NADPH-oxidase ($F1, 21 = 0.1419$, $p = 0.7102$). There was no significant interaction between MPTP and diet ($F1, 21 = 1.128$, $p = 0.3002$). The *post-hoc* test showed that MPTP treatment alone significantly suppressed the mRNA expression of the NADPH-oxidase $p47^{phox}$ when compared to the effect in the control group ($t = 2.611$, $p<0.05$) (Figure 3.4.1A).

However, the two-way ANOVA indicated that neither the MPTP factor ($F1, 20 = 0.8166$, $p = 0.3769$) nor the diet factor ($F1, 20 = 0.1336$, $p = 0.7186$) had any significant effect on the mRNA expression of the pro-oxidant enzyme iNOS. There was also no interaction between

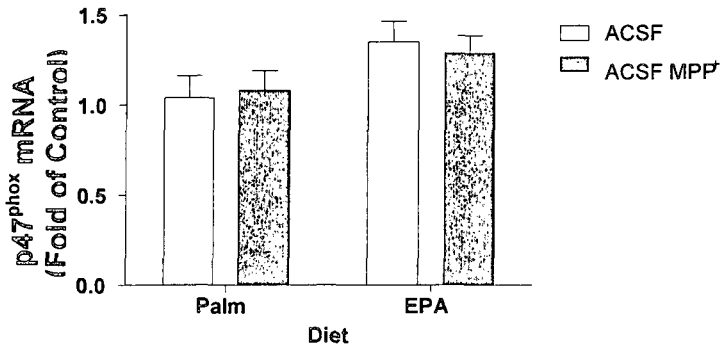

Figure 3.3.1. The effect of MPP^+ and EPA on mRNA expressions of the pro-oxidant enzyme in the brain slice model: MPP^+ treatment effects on the mRNA expressions of the pro-oxidant enzyme NADPH-oxidase $P47^{phox}$ in the striatal slices of the PD model with an EPA or a palm oil diet (control), analyzed by quantitative PCR. The results of the PCR analysis are presented as the fold of control, and statistically expressed as mean ± SEM (C: n = 6; E: n = 6; M: n = 6; EM: n = 8). C, Palm oil diet and ACSF; E, EPA diet and ACSF; M, Palm oil diet and ACSF with MPP^+, and EM, EPA diet and ACSF with MPP^+.

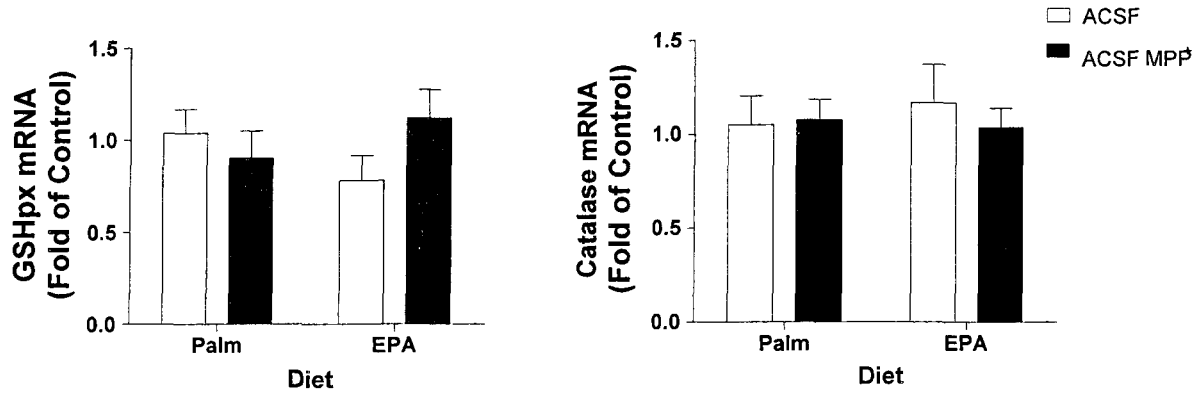

Figure 3.3.2. The effect of MPP^+ and EPA on mRNA expressions of the antioxidant enzymes in the brain slice model: MPP^+ treatment effects on mRNA expressions of antioxidant enzymes in striatal slices of the PD model with an EPA or a palm oil diet (control), measured by quantitative PCR and presented as the fold of control. The results of the PCR test are expressed as mean ± SEM. (A) mRNA expressions of GSHpx in striatal slices of mouse brains under the effect of MPP^+ with or without an EPA diet were tested (C: n = 6; E: n = 5; M: n = 6; EM: n = 7). (B) mRNA expressions of catalase in striatal slices of mouse brains under the effect of MPP^+ with or without an EPA diet were tested (C: n = 6; E: n = 6; M: n = 6; EM: n = 8). C, Palm oil diet and ACSF; E, EPA diet and ACSF; M, Palm oil diet and ACSF with MPP^+, and EM, EPA diet and ACSF with MPP^+.

MPTP and diet either (F1, 20= 0.8971, p = 0.3549) (One outlier was removed from MPTP with EPA group) (Figure 3.4.1B).

With respect to antioxidant enzymes, the two-way ANOVA revealed that neither the MPTP factor nor the diet factor had a significant effect on mRNA expressions of the antioxidant enzymes SOD (MPP^+: F1, 24 = 0.7141, p = 0.4064; diet: 1, 24 = 3.228, p = 0.0850) and GSHpx (MPP^+: F1, 24 = 0.2428, p = 0.6266; diet: F1, 24 = 1.215, p = 0.2812). No significant interaction between MPP^+ and diet in SOD was observed (F1, 24 = 0.9439, p = 0.3410), while there was an interaction between MPP^+ and diet for GSHpx (F1, 24 = 7.131, p = 0.0134) (Figure 3.4.2 A and B).

However, the two-way ANOVA showed that the effect of the MPTP factor on the mRNA expression of catalase approached significance (F1, 23 = 3.85, p = 0.0618), while the diet factor had no effect on the mRNA expression of catalase (F1, 23 = 0.3208, p = 0.5766). There was a significant interaction between MPTP and diet (F1, 23 = 5.36, p = 0.0298). The *post-hoc* test showed that MPTP treatment alone significantly decreased the mRNA expression of catalase when compared to the results obtained in the control group (t = 2.775, $p<0.05$). EPA treatment alone had no significant effect on the mRNA expression of catalase when compared to the control group. In the group administered both EPA and MPTP, the mRNA expression of catalase was not significantly different from the group treated with MPTP alone. However, this difference between the group treated with MPTP alone and the group treated with EPA combined with MPTP was significant after analysis by an unpaired t-test (t = 2.123, $p<0.05$) (One outlier was removed from MPTP group) (Figure 3.4.2 C).

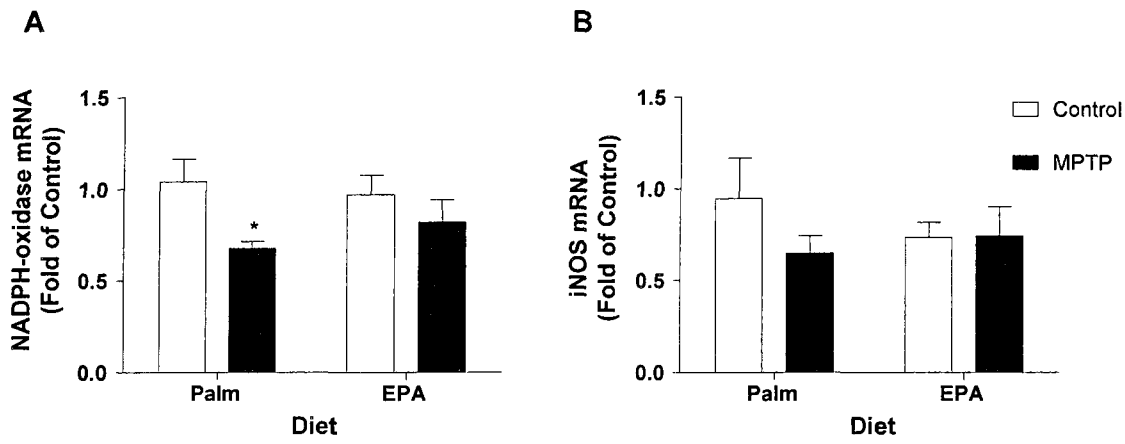

Figure 3.4.1. The effect of MPTP and EPA on mRNA expressions of the pro-oxidant enzymes in the midbrain of the chronic mouse model: MPTP effects on mRNA expressions of pro-oxidant enzymes in the midbrain of the chronic PD model mouse with an EPA or a palm oil diet (control), as analyzed by quantitative PCR and presented as the fold of control. The results of PCR analyses are expressed as mean ± SEM. (A) the mRNA expressions of the NADPH-oxidase $P47^{phox}$ in mouse midbrains under the effect of MPTP with or without an EPA diet was tested (C: n = 6; E: n = 6; M: n = 7; EM: n = 6). (B) mRNA expressions of iNOS in midbrain under the effect of MPTP with or without an EPA diet were tested (C: n = 7; E: n = 6; M: n = 6; EM: n = 5). * $p<0.05$ as compared to the control. C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

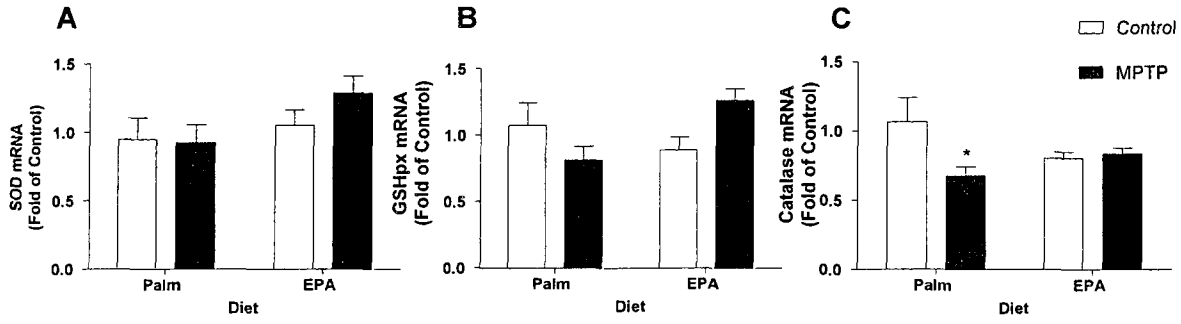

Figure 3.4.2. The effect of MPTP and EPA on mRNA expressions of the antioxidant enzymes in the midbrain of the chronic mouse model: MPTP effects on mRNA expressions of antioxidant enzymes in the mouse midbrains of chronic PD model with an EPA or a palm oil diet (control), measured by quantitative PCR and presented as the fold of control. The results of PCR analyses are expressed as mean ± SEM. (A) mRNA expressions of SOD in the midbrain of mice under the effect of MPTP with or without an EPA diet were tested (C: n = 6; E: n = 8; M: n = 6; EM: n = 8). (B) mRNA expressions of GSHpx in the midbrain of mice under the effect of MPTP with or without an EPA diet were tested (C: n = 7; E: n = 8; M: n = 6; EM: n = 7). (C) mRNA expressions of catalase in the midbrain under the effect of MPTP with or without an EPA diet were tested (C: n = 6; E: n = 8; M: n = 5; EM: n = 8). * $p<0.05$ as compared to the control. C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

3.3.5 *mRNA expressions of oxidative and antioxidant enzymes in the striatum of chronic PD model*

To test the interrelation between pro-oxidant and antioxidant enzymes in the striatum of the chronic PD model, quantitative PCR was used. This experiment contained four groups, which were the same as the fatty acid analysis groups in the chronic model in the Section 3.3.2. For pro-oxidant enzymes, the two-way ANOVA showed that neither the MPTP factor ($F1, 19 = 0.02487$, $p = 0.8788$) nor the diet factor ($F1, 19 = 0.3120$, $p = 0.5830$) had a significant effect on the mRNA expressions of the NADPH-oxidase $P47^{phox}$. However, there was a significant interaction between MPTP and diet ($F1, 19 = 6.05$, $p = 0.0236$) (Figure 3.5.1A).

Furthermore, the two-way ANOVA indicated that the effects of the MPTP factor on the mRNA expression of the pro-oxidative enzyme iNOS approached significance ($F1, 20 = 3.48$, $p = 0.0768$), while the diet factor had no effect on the mRNA expression of iNOS ($F1, 20 = 0.7567$, $p = 0.3947$). There was no significant interaction between MPTP and diet ($F1, 20 = 2.478$, $p = 0.1311$) (One outlier was removed from MPTP group) (Figure 3.5.1B).

For the antioxidants in the striatum of this chronic PD model, a two-way ANOVA revealed that the MPTP factor did not significantly affect the mRNA expressions of SOD ($F1, 24 = 0.1537$, $p = 0.6985$) and catalase ($F1, 24 = 0.6471$, $p = 0.4291$), and the diet factor had no effect on the mRNA expressions of SOD ($F1, 24 = 2.788$, $p = 0.1080$) and catalase ($F1, 24 = 0.7984$, $p = 0.3804$). Neither was there an interaction between MPTP and diet (SOD: $F1, 24 = 1.429$, $p = 0.2436$; catalase: $F1, 24 = 0.2040$, $p = 0.6556$) (Figure 3.5.2 A and C).

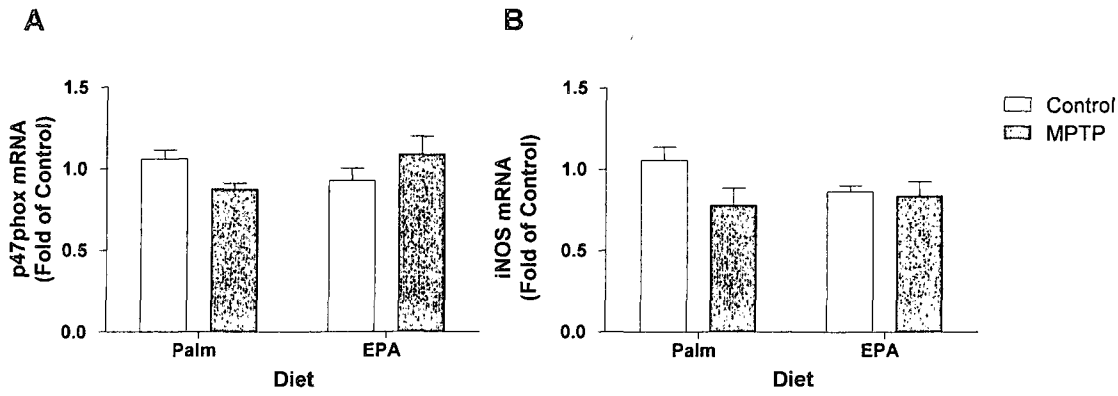

Figure 3.5.1. The effect of MPTP and EPA on mRNA expressions of the pro-oxidant enzymes in the striatum of the chronic mouse model: MPTP effects on mRNA expressions of pro-oxidant enzymes and antioxidant enzymes in the striatum of the chronic MPTP mouse model fed with an EPA or a palm oil diet (control), measured by quantitative PCR and presented as the fold of control. The results of PCR analyses are expressed as mean ± SEM. (A) mRNA expressions of the NADPH-oxidase $P47^{phox}$ in the mouse striatum under the effect of MPTP with or without an EPA diet were tested (C: n = 6; E: n = 6; M: n = 6; EM: n = 5). (B) mRNA expressions of iNOS in the mouse striatum under the effect of MPTP with or without an EPA diet were tested (C: n = 7; E: n = 6; M: n = 5; EM: n = 6). C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

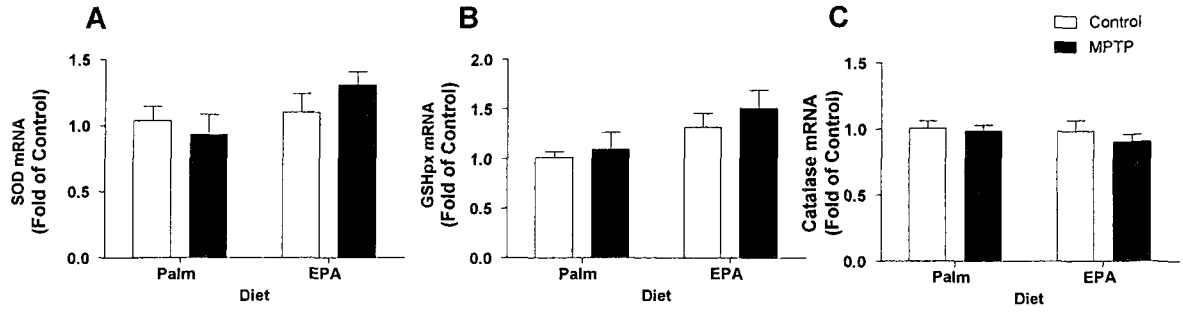

Figure 3.5.2. The effect of MPTP and EPA on mRNA expressions of antioxidant enzymes in the striatum of the chronic mouse model: MPTP effects on mRNA expressions of antioxidant enzymes in the striatum of the chronic MPTP mouse model fed an EPA or a palm oil diet (control), measured by quantitative PCR and presented as the fold of control. The results of PCR analyses are expressed as mean ± SEM. (A) mRNA expressions of SOD in the mouse striatum under the effect of MPTP with or without an EPA diet were tested (C: n = 8; E: n = 8; M: n = 6; EM: n = 6). (B) mRNA expressions of GSHpx in the mouse striatum under the effect of MPTP with or without an EPA diet were tested (C: n = 7; E: n = 7; M: n = 6; EM: n = 6). (C) mRNA expressions of catalase in the mouse striatum under the effect of MPTP with or without an EPA diet were tested (C: n = 8; E: n = 8; M: n = 6; EM: n = 7). C, Palm oil diet and control treatment; E, EPA diet and control treatment; M, Palm oil diet and MPTP treatment, and EM, EPA diet and MPTP treatment.

As well, the two-way ANOVA showed that the MPTP factor did not significantly affect the mRNA expression of GSHpx ($F1, 21 = 0.9334$, $p = 0.3443$), while the diet factor had a significant effect on the mRNA expression of GSHpx in the striatum of the chronic PD model ($F1, 22 = 6.633$, $p = 0.0173$). There was no interaction between MPTP and diet ($F1, 21 = 0.1558$, $p = 0.6968$). The *post-hoc* test also showed that the mRNA expression of GSHpx in the group treated with EPA alone group was no different from that in the control group. In the group treated with EPA combined with MPTP, the mRNA expression of GSHpx was not significantly different from that in the MPTP-only group (Figure 3.5.2B).

3.4 Discussion

With the brain slice model of PD, the present study demonstrated that: 1) in the fatty acid profile, MPP^+ increased the concentrations of LA and AA without affecting n-3 fatty acids in the brain slice tissues when compared to the control. EPA treatment alone significantly increased EPA and DPA concentrations, while having no effect on the concentration of other fatty acids in the brain slice tissues. In the group treated with both MPP^+ and EPA, EPA attenuated the effect of MPP^+ on LA and AA concentrations when compared to MPP^+ treatment alone. In the EPA and MPP^+ treatment groups, an EPA-enriched diet also significantly increased both EPA and DPA concentrations when compared to the MPP^+ treatment alone and 2) with respect to mRNA expressions of enzymes, MPP^+ treatment alone had no effect on the mRNA expressions of the pro-oxidant enzyme NADPH-oxidase and the antioxidant enzymes catalase and GSHpx. As well, EPA treatment alone also had no effect on the pro-oxidant and antioxidant enzymes when compared to the control. The

pro-oxidant and antioxidant enzymes in the group with both EPA and MPP^+ treatments were no different from the group with MPP^+ treatment alone either.

In the chronic PD model, the present study has demonstrated that: 1) with respect to the concentration of fatty acids, MPTP treatment alone had no effect on the concentrations of n-3 fatty acids and n-6 fatty acids when compared to the control group. When compared to the control, EPA treatment alone significantly increased EPA and DPA concentrations, while having no effect on any other fatty acids. EPA and DPA concentrations in the group with both MPTP and EPA treatments were also increased when compared to the group with MPTP treatment alone; 2) with respect to mRNA expression, in the midbrain of the chronic mouse model, MPTP significantly decreased the mRNA expression of the pro-oxidant enzyme NADPH-oxidase $p47^{phox}$, while having no effect on mRNA expression of iNOS. Additionally, when compared to the control, MPTP significantly decreased the mRNA expression of the antioxidant enzyme catalase while having no effect on the mRNA expressions of the other antioxidant enzymes, SOD and GSHpx. EPA treatment alone had no effect on mRNA expressions of the pro-oxidant and antioxidant enzymes when compared to the control. In the group with both EPA and MPTP treatments, mRNA expressions of the pro-oxidant and antioxidant enzymes showed no significant difference from those in the group administered MPTP treatment alone, and 3) in the striatum of the chronic PD model, MPTP treatment alone had no effect on pro-oxidant enzymes and antioxidant enzymes when compared to the control group. EPA treatment alone had no effect on the mRNA expressions of the pro-oxidant enzymes and antioxidant enzymes when compared to the control group either. Similar results were found in the group with

both EPA and MPTP treatments. There was no difference between mRNA expressions of the pro-oxidant enzymes and antioxidant enzymes in the EPA and MPTP combined group and MPTP treatment alone group. According to these results, the discussion is presented below.

3.4.1 MPTP effects on the n-3 fatty acids and n-6 fatty acids

In the brain slice study, MPP^+ in the acute model significantly increased the concentrations of the n-6 fatty acids LA and AA in the brain tissue, but not the n-3 fatty acids. To date, there has been no study reporting fatty acid changes in mice treated with acute MPP^+. These results, for the first time, showed that acute MPP^+ may change the biosynthesis of n-6 fatty acids and produce more n-6 fatty acids in the brain. As mentioned in Section 1.6.3, Chapter 1, the n-6 fatty acid AA and its precursor LA can form as free fatty acids through fatty acid metabolism. AA is a precursor of the PGE2 and other inflammatory mediators under the action of enzyme cytosolic phospholipase a2 (cPLA2). Therefore, an increase in AA productions may cause inflammation, which would then trigger oxidants that damage neurons (Bagga *et al.*, 2003). Increased AA and LA induced by MPP^+ may explain the previous finding that cPLA2 and PGE2, which are related to AA metabolism, are involved in MPP^+-induced neurodegeneration (Wang *et al.*, 2005; Tariq *et al.*, 2001). The mechanism by which MPP^+ affects n-6 fatty acids biosynthesis is unclear, and it should be further investigated.

Previous studies demonstrated that a high intake of saturated fats and a low intake of unsaturated fats may increase the risk of PD development (Logroscino *et al.*, 1996; Johnson

et al., 1999; Anderson *et al.*, 1999), which indicates a strong relationship between PD onset and unsaturated fats. However, the present study did not find that MPTP significantly changed the concentrations of the unsaturated n-6 fatty acids LA and AA, nor did it affect any other n-3 and n-6 fatty acids in the chronic study. Similar results in the fatty acid profile after chronic MPTP administrations were found in monkeys and in the postmortem cortex of PD patients (Julien *et al.*, 2006). By contrast, after acute MPP^+ administration, increased concentrations of AA and its precursor LA were found in the present study. The reason for this difference could be that, as mentioned in Section 1.6.3, Chapter 1, AA is the major material for the precursor of several inflammatory mediators. In the chronic model, LA and AA may be released and used to produce PGE2 and other inflammatory mediators, which cannot be detected in its fatty acid form (Bagga *et al.*, 2003). Therefore, the increases in the concentrations of LA and AA observed in the acute model are reduced in the chronic model.

3.4.2 MPTP effects on pro-oxidant enzymes and antioxidant enzymes

In the acute MPTP model (brain slice study), MPTP did not affect mRNA expressions of pro-oxidant and antioxidant enzymes. This result may suggest that acute MPTP treatments were not sufficient to cause changes in the pro-oxidant and antioxidant enzymes at the mRNA level, while in the midbrain of the chronic mouse model in the present study, a decreased mRNA expression of NADPH-oxidase was found. The dopaminergic neuron loss induced by chronic $MPTP^+$ may destroy the enzymes in the neurons. Thus, the decreased NADPH-oxidase may indicate dopaminergic neuron loss. This seems to contradict earlier findings, as Wu and colleagues reported that the mRNA expression of NADPH-oxidase in

mice with 14 days of MPTP injections was increased in midbrain (Wu *et al.*, 2003). The difference may be due to the different time courses of MPTP treatments. As mentioned in Section 1.5.3.1, Chapter 1, the time course of MPTP treatments may induce different effects on neuron survival and death, which may reflect different effects of oxidative stress on cell functions. With regards to antioxidant enzymes, catalase was only suppressed in the midbrain (containing the SN) but not in the striatum in the chronic MPTP model, which indicates that the antioxidant defense mechanism was somehow reduced by MPTP. This result is in line with studies with PD patients and a mouse PD model, which found that the antioxidant catalase defense is reduced in the parkinsonian SN (reviewed by Fahn and Cohen, 1992; Ambani *et al.*, 1975). The reason why the decrease in mRNA expressions of the pro-oxidant enzyme NADPH-oxidase and the antioxidant enzyme catalase were only found in the midbrain but not in the striatum could be dopaminergic neurons mainly existing in the SN of the midbrain (reviewed by Chinta and Andersen, 2005). Since the SN also contains the microglia with highest density (reviewed by Rogers *et al.*, 2007), the activation of microglia in response to MPTP treatment may contribute to dopaminergic neuron death in PD. Furthermore, dopaminergic cell loss can also induce a decrease in the enzymes of the cells. Therefore, in the present study, both decreases in the pro-oxidant and antioxidant enzymes may indicate dopaminergic neuron loss in the midbrain.

3.4.3 EPA effects on the n-3 fatty acids and n-6 fatty acids

A diet of EPA alone increased the concentrations of the n-3 fatty acids EPA and DPA without changing the n-3 fatty acid DHA, in the brain tissues in both studies. These results demonstrated that an EPA-enriched diet could modify n-3 fatty acid contents in the mouse

brain, which confirmed that changes in the mammalian brain fatty acid profile depend on dietary intake (Pawlosky *et al.*, 2001; Moriguchi *et al.*, 2004). However, EPA intake did not significantly increase DHA contents in the mouse brain, which confirmed a study reporting that EPA may not readily convert to DHA (Sargent *et al.*, 1999). The reason may be due to an insufficient amount of the transforming enzyme that controls the conversion of EPA to DHA. Furthermore, the important finding that DPA concentrations were significantly increased by the EPA diet may suggest that DPA may be involved in many aspects of EPA biofunctions. Further study should explore this direction.

3.4.4 EPA effects on the antioxidant enzymes and pro-oxidant enzymes

EPA, as an antioxidant and a component of cell membranes, can prevent oxidants, and thus could prevent oxidative stress in the brain. The n-3 fatty acids DHA and EPA can attenuate oxidative stress and NO, which may have a positive regulating role in the synthesis of antioxidant enzymes such as SOD and GSHpx (Li *et al.*, 2006). DPA is an intermediary fatty acid between EPA and DHA, while its function in the brain is still unclear. In the present study, EPA treatment alone did not affect the pro-oxidant enzymes and antioxidant enzymes in brain slice study and the chronic study. Thus EPA treatment alone may not affect normal levels (without MPTP/MPP^+ insult) of pro-oxidant enzymes and antioxidant enzymes.

3.4.5 The effect of EPA combined with MPTP on the n-3 fatty acids and n-6 fatty acids

In the group treated with both EPA and MPTP, the EPA and DPA concentrations were also increased in the brain tissues in both studies. These results indicate that MPP^+ did not

interact or interfere with EPA effects on n-3 fatty acid construction in the both acute and chronic models. In addition, this study has been the first to demonstrate that EPA attenuates the effect of MPTP on n-6 fatty acids LA and AA, but only in the acute model (in brain slice study), which confirmed that EPA can compete with AA with respect to incorporation into cell membrane phospholipids (reviewed by Lands, 1992). As mentioned in Section 1.6.3, Chapter 1 and above, AA can form as free fatty acids that produce PGE2 and other proinflammatory mediators under the action of enzyme cPLA2. Thus, EPA may benefit brain functions through decreasing AA concentrations and reducing the risk of inducing inflammation.

3.4.6 The effect of EPA combined with MPTP on pro-oxidant enzymes and antioxidant enzymes

In the acute model, there were no significant differences in mRNA expressions of these enzymes in the group with both EPA and MPTP treatments as compared to the group with MPTP treatment alone. As discussed in Section 3.4.2, acute MPTP may not cause any effect on pro-oxidant and antioxidant enzymes. Thus, without significant effects from MPTP treatment, EPA may not affect these enzymes at the baseline. As well, in the chronic PD model, EPA did not attenuate the effect of MPTP on the mRNA expression of NADPH-oxidase. However, EPA marginally attenuated the MPTP-induced decrease in the mRNA expression of catalase in the midbrain of the chronic MPTP model. Catalase, as an antioxidant enzyme, has detoxifying effects, as it reacts with the oxidant H_2O_2 to produce water and oxygen (reviewed by Özben, 1998). In the chronic model, the antioxidant defense system had been suppressed by the effect of chronic MPTP administration, which

induced a decrease in catalase. As well, this study is the first to show that EPA has the potential to attenuate the effect of MPTP on the antioxidant defense system, since EPA marginally attenuated the MPTP-induced decrease in the mRNA expression of catalase.

It should be discussed that although EPA treatment had no significant effect on pro-oxidant enzymes and antioxidant enzymes in the mice treated with MPTP (as shown by the post-hoc test), the two-way ANOVA indicated that the diet factor had a significant effect on the antioxidant enzyme GSHpx in the striatum. As well, the effect of EPA on the decrease in the mRNA expression of catalase induced by MPTP approached significance (by the two-way ANOVA with a host-hoc test) in the midbrain. The nonsignificant result may be caused by insufficient feeding time or the small sample size. Nevertheless, both of them showed that EPA has the potential to up-regulate the anti-oxidative system against oxidant production.

Taken together, the EPA was successfully incorporated into the brain, changed n-3 and n-6 fatty acid profiles, and attenuated the effect of MPTP on antioxidant enzymes. However, its failure to change a number of significant markers suggested that the duration of EPA treatments should be extended, which may help EPA efficacy.

In summary, MPTP increased the concentrations of potential inflammatory triggers, the n-6 fatty acids LA and AA, in the acute PD model, and suppressed the pro-oxidant enzyme NADPH-oxidase $p47^{phox}$ and the antioxidant enzyme catalase in the chronic PD model. EPA increased concentrations of the n-3 fatty acids EPA and DPA in both the acute and

chronic PD models at baseline as well as under MPP^+ treatment, and also increased concentrations of EPA and DPA in both the acute and chronic PD models. In the acute PD model, EPA attenuated the MPTP/MPP^+-induced increase in n-6 fatty acids LA and AA, which are potential inflammatory triggers. However, EPA could not significantly attenuate the MPP^+-induced decrease in the pro-oxidant enzyme NADPH-oxidase $p47^{phox}$ and the antioxidant enzyme catalase in the chronic model, which may suggest that MPTP/MPP^+-induced changes in the oxidative and anti-oxidative system are not directly related to the effect of MPTP/MPP^+ on n-3 and n-6 fatty acids.

Chapter 4

General Discussion

4.1 Overview

In this thesis, MPTP/MPP^+ was used to induce three PD models: an *in vitro* study with cell cultures and two animal models, including acute and chronic models. In the *in vitro* study, MPTP/MPP^+ decreased mitochondrial dehydrogenase activity, which was determined by MTT assay. To investigate whether oxidative stress was involved in this model, the oxidative system and anti-oxidative systems were studied. In the oxidative system, oxidant production was measured by the fluorescent DCF-DA for ROS and the fluorescent DAF-2DA for RNS. It was found that MPTP/MPP^+ enhanced the production of the cellular oxidant ROS, presumably induced by an increased expression of the enzyme NADPH-oxidase. The expressions of the NADPH-oxidase $p47^{phox}$ at the mRNA and protein levels were measured by quantitative PCR and Western blot. NO, a RNS, was also found to be increased by MPTP/MPP^+, whereas neuronal nNOS mRNA expression was decreased, as tested by quantitative PCR. The changes in the anti-oxidative system in response to MPTP/MPP^+ insult were also evaluated. The mRNA expression of antioxidant enzymes was tested by quantitative PCR as well, while the concentration of the antioxidant GSH was measured by a GSH assay kit. It was found that in response to oxidative activation, the concentration of the antioxidant GSH and mRNA expressions of antioxidant enzymes SOD, catalase and GSHpx were increased. Next, dietary effects on the fatty acid profile and the relationship between fatty acids and oxidative and anti-oxidative changes were explored. In the brain slice model of PD, MPTP/MPP^+ increased the n-6 fatty acids LA and AA in the midbrain and frontal cortex, as measured by GC. However, MPTP/MPP^+ did not affect the oxidative and anti-oxidative systems at the mRNA level, since there were no changes in

mRNA expressions of the pro-oxidant enzyme NADPH-oxidase and the antioxidant enzymes catalase and GSHpx.

In the chronic PD model, MPTP/MPP^+, when administered independently, had no effect on the concentrations of n-3 and n-6 fatty acids in the frontal cortex. In the midbrain, MPTP/MPP^+ significantly decreased the mRNA expression of the antioxidant enzyme catalase, whereas it had no effect on mRNA expressions of the other antioxidants, SOD and GSHpx. Additionally, MPTP/MPP^+ significantly decreased the mRNA expression of the NADPH-oxidase $p47^{phox}$, while having no effect on iNOS mRNA expression. In the striatum of the chronic model, MPTP/MPP^+ alone had no effect on pro-oxidant enzymes or antioxidant enzymes.

In addition to creating PD models and studying oxidative mechanisms, a new therapy for PD, the n-3 fatty acid EPA, was administered to the three PD models. In the *in vitro* study, EPA alone (in the control condition without MPTP/MPP^+) up-regulated mitochondrial dehydrogenase activity and down-regulated the mRNA expression of nNOS, while displaying no other significant effects on the oxidative and anti-oxidative systems.

In the brain slice experiment, EPA treatment alone significantly increased concentrations of the n-3 fatty acids EPA and DPA. However, EPA had no effect on the biomarkers of oxidative and anti-oxidative systems at the mRNA level, such as mRNA expressions of catalase, GSHpx and NADPH-oxidase.

In the chronic model, EPA treatment also significantly increased the concentrations of the n-3 fatty acids EPA and DPA, but lacked effects on other fatty acids. EPA alone had no effect on mRNA expressions of the pro-oxidant enzymes NADPH-oxidase and iNOS, nor the antioxidant enzymes SOD, catalase and GSHpx, in either the mouse midbrain or striatum.

In the *in vitro* model, EPA reversed the MPTP/MPP^+-induced reduction of mitochondrial dehydrogenase activity and down-regulated MPTP/MPP^+-induced increases in ROS and RNS productions. EPA also attenuated the effects of MPTP/MPP^+ on mRNA and protein expressions of NADPH-oxidase and on the mRNA expression of the antioxidant enzyme GSHpx. However, EPA had no significant effect on the mRNA expression of the pro-oxidant enzyme nNOS, the antioxidant enzymes SOD and catalase, or the concentration of the antioxidant GSH under MPTP/MPP^+ insult.

In the brain slice study, EPA decreased the MPTP/MPP^+-induced increase in concentrations of n-6 fatty acids (LA and AA), and significantly increased concentrations of n-3 fatty acids (EPA and DPA) in the MPTP/MPP^+-treated brain slices of the PD model. However, EPA did not have any effects on the oxidative and anti-oxidative systems at the mRNA level, since EPA did not change the effect of MPTP/MPP^+ on mRNA expressions of catalase, GSHpx and NADPH-oxidase.

In the chronic MPTP/MPP^+-induced PD model, EPA also significantly increased concentrations of n-3 fatty acids (EPA and DPA). However, EPA was unable to attenuate

the effect of MPTP/MPP^+ on the oxidative and anti-oxidative systems at the mRNA level, as measured by mRNA expressions of the antioxidants SOD, catalase and GSHpx, and mRNA expressions of the pro-oxidants enzymes NADPH-oxidase and iNOS, in the both midbrain and striatum.

As summarized above, MPTP/MPP^+-induced changes in oxidative and anti-oxidative systems, as well as in the fatty acid profile after MPTP/MPP^+ administrations, were varied across the three different models. The effects of EPA on these systems and on the fatty acid profile in normal and MPTP/MPP^+-treated conditions were also different among the models. Therefore, the mechanisms by which MPTP/MPP^+ and EPA differently affected these parameters are addressed in Section 4.3.

As several research methods were selected to study the oxidative stress and the fatty acid profile in the different models of PD, the methodology, including an introduction to the choice of disease models and their advantages and disadvantages, is discussed in Section 4.2.

4.2 Methodology

4.2.1 Cell culture

Human SH-SY5Y dopaminergic neuroblastoma cells were used in this study. SH-SY5Y cells are considered to be suitable for an *in vitro* model of PD, and have been utilized in many studies with MPTP administrations (Cassarino *et al.*, 1997; Chen *et al.*, 2005; Dennis and Bennett, 2003; Kou *et al.*, 2008). Firstly, aside from being human neuroblastoma, these

cells can be cultured in large quantities, thereby providing high rates of reproducibility. Secondly, SH-SY5Y cells are a good model for long-term experiments, as they can survive for several generations and are easy to get and culture, compared to human primary neurons. Although SH-SY5Y neuroblastoma cells have many advantages, they are not true neurons. Thus, the combination of RA and TPA treatments was selectively utilized in this study to enhance the DA transport system in the cells, and make the cells more neuron-like, and increasing their sensitivity to MPTP/MPP^+ (Presgraves *et al.*, 2004). Additionally, to increase uniformity of the cells, the cultured SH-SY5Y cells were controlled from generation 20 to 30. The incubation time with different treatments was controlled from 2 to 3 days. The morphology of the cells was observed daily, and according to the morphological criteria, only when cell differentiation was qualified would they be used in the study (Lanciotti *et al.*, 1991; Presgraves *et al.*, 2004).

4.2.2 MTT assay

Following different treatments, mitochondrial dehydrogenase activity was measured by MTT assay. MTT is used as a measure of mitochondrial activity in a colorimetric assay to test the activation level of cells via mitochondrial dehydrogenase (Gerlier, 1986). Thus, results gained from this study only indicated the effect of treatment on the activation of cell mitochondria, but it is not directly indicative of cell death.

4.2.3 DCF-DA test

After obtaining the results from the MTT assay, oxidant production was tested by DCF-DA and DAF-2DA. The fluorescent dye DCF-DA is a useful tool for measuring intracellular

H_2O_2 contents (Oyama *et al.*, 1994), as DCF-DA is very sensitive to ROS that reflect a part response during oxidative stress. However, the DCF-DA fluorescence for testing the H_2O_2 amount was not very stable, which caused some differences between tests. As fluorescence is light-sensitive, samples should always be covered by a lightproof lid. The changeable results may have been induced by lighting. Other factors may also contribute to the changeable results, such as differing incubation times, or differing cell conditions, including poorly differentiated cells, or cells in the midst of dividing. To increase replicability, the same incubating duration was always maintained to prevent variablity in results from separate tests.

4.2.4 Quantitative PCR

In the present study, pro-oxidant enzymes and antioxidant enzymes, which may affect the oxidants, were tested at mRNA levels by quantitative PCR. Quantitative PCR is a very efficient tool to test gene expressions (mRNA expressions). Quantitative PCR is applied to quantified gene expressions rather than qualified. It is more sensitive and accurate than regular PCR. Due to the low amount of mouse brain tissues in the brain slice and chronic PD models, RNA amounts extracted from mouse brain tissues were very low, and may have been insufficient to detect the target genes. The sample size was also variable for the same reason. Since the number of samples was reduced, the results may have had larger variations and a higher possibility of non-significant results.

4.2.5 Gas chromatography

To analyze the fatty acid profile in brain tissues, GC was used. It is first time that this GC method was used in this lab to measure n-3 and n-6 fatty acids in both mouse and mouse brain tissues. This fatty acid analysis method can provide direct evidence as to whether an EPA diet affects the profiles of n-3 and n-6 fatty acids, and reveal the relationship between changes in fatty acids and oxidative stress in the brain.

When trying to set up the GC, difficulties were encountered in selecting internal standards. The internal standard requires a fatty acid that is detectable by this method, but which is not contained in the brain tissues. In theory, fatty acids with odd numbers of carbon atoms do not exist in mammalian tissue. However, a small amount of the fatty acids C15:0, C17:0, C19:0, C21:0 and C23:0 were detected in the brain tissue samples. Thus, none of those fatty acids could be used as the internal standard. Eventually, C13:0 was selected as the internal standard, as this fatty acid was not contained in the samples and its peak was not shown in the main area of the target peaks (from C18 to C22). Therefore, C13:0 would not affect the accuracy of results.

The results obtained through these methods in three PD models will be discussed as follows.

4.3 Result discussion

Oxidative stress plays an important role in the pathogenesis of PD (reviewed by Jenner *et al.*, 1992). The present study sought to investigate the interrelation between the oxidative

and the anti-oxidative systems in PD, and to evaluate EPA therapy in three models: the cellular model and the systematic models, the latter involving both acute and chronic models. The cellular mechanisms of oxidative stress in the neuronal model of PD and the neuroprotective effects of EPA against oxidative damage were studied as well. Furthermore, the systematic mechanism of oxidative stress in PD and the effects of EPA therapy on PD were studied with animal models, including the acute PD model (brain slice study) and chronic PD model. The systematic model was used not only for testing the oxidative stress mechanism of neurons in the brain, but also for testing the oxidative stress mechanism of entire brain system in the animals, including in the both neurons and glial cells. The results in these three models under the MPTP/MPP^+ administration, EPA treatment, and MPTP/MPP^+ combined with EPA treatments will be discussed below.

PD is a progressive degenerative disease in the CNS, characterized by the loss of dopaminergic neurons (Antoniades and Barker, 2008). In PD, oxidative stress is considered as one contributor to dopaminergic cell death (reviewed by Jenner *et al.*, 1992). This study also confirmed this theory, since the oxidants H_2O_2 and NO were both increased in the MPP^+ treated cellular model, which is consistent with other *in vitro* studies (Kalivendi *et al.*, 2003). As mentioned in the introduction, the pro-oxidant enzyme, NADPH-oxidase is one of the main sources of ROS (Förstermann *et al.*, 1995), which may be the one of the major triggers of increased oxidants in PD. The result of this study showed that NADPH-oxidase was increased by MPP^+ in the cellular model, which is consistent with previous findings in the brains of PD patients (Wu *et al.*, 2003). In addition, a decrease in this enzyme was

found in the midbrain of the chronic model, which may possibly indicate that neuron loss induced the enzyme deactivation at the late stage.

Oxidative stress can be induced when the body is unable to readily detoxify oxidants and repair damage (reviewed by Mena *et al.*, 2008). Because antioxidants can prevent the damage induced by oxidants, antioxidants also play a key role in the oxidative stress mechanism. However, the results that were found in the cellular model were different from the hypothesis of this thesis, which was that antioxidant enzymes would be decreased under MPTP treatment. The fact is that antioxidants SOD, catalase and GSHpx, were all increased under MPP^+ treatment in the cellular model, where they may exert a protective effect on neurons. However, the same result did not occur in the two animal models.

It is important to note here, as mentioned in Section 1.4.4.1, Chapter 1, that mitochondrial dysfunction may result in an over-production of oxidants in PD (reviewed by Przedborski and Vila, 2003). Indeed, in the cellular model of this study, mitochondrial dehydrogenase activity was decreased by MPTP, which may be related to the increased oxidant production induced by MPTP. Moreover, it has been reported that oxidative stress is associated with lipid peroxidation (which may reduce fatty acid contents) in the brain (reviewed by Trushina and McMurray, 2007). However, this thesis did not find any decreased fatty acids in mouse brains, at the acute stage, while n-6 fatty acids were increased by MPTP/MPP^+ treatment. MPTP increased n-6 fatty acid content in the brain by modifying the n-6 fatty acid biosynthesis processes. N-6 fatty acids can potentially produce inflammatory triggers, which may cause neuron death (Bagga *et al.*, 2003a).

As mentioned in the introduction, there is currently no fully effective treatment for PD patients and most existing treatments have adverse side effects. N-3 fatty acids act as a functional food to defend against neurodegenerative diseases (reviewed by Calon and Cole, 2007). N-3 fatty acids may increase DA levels, down-regulate oxidative stress, and prevent PD in the *in vivo* model (Yehuda, 2002). EPA, an n-3 fatty acid, has shown neuroprotective, antioxidant and anti-inflammation functions in many other neurodegenerative disease models (Schmitz and Ecker, 2008; Richard *et al.*, 2008). In this study, dietary EPA fed for 6 weeks was successfully transformed into brain lipids, which provided evidence for the future study of EPA effects on PD animal models. In the cellular model of this study, EPA modulated oxidative stress by reducing NADPH-oxidase and the MPTP/MPP^+-induced increase in oxidant levels, and up-regulated decreased mitochondrial activity. However, EPA treatment did not significantly modulate oxidative stress induced by MPTP in the animal models, even though EPA was successfully transferred to the brain. This may indicate that MPTP/MPP^+ presumablly takes a shorter time to cause damage, while EPA may need a longer time to change brain function.

Overall, in this study, the three models provided complementary results, revealing different mechanisms involved in MPTP/MPP^+-induced changes and provided many new findings for further study in PD. The insights of these differences will be discussed below.

4.3.1 MPTP/MPP^+-induced changes in the oxidative and anti-oxidative systems, and fatty acid profile in the different models

In the cellular model, differentiated SH-SY5Y cells were directly incubated with media containing 0, 50 or 100 μM MPP^+ for 48 or 96 hours. In the acute animal model (the brain slice model), striatal slices were directly incubated in 20 μM MPP^+ ACSF for 4 hours. In the chronic animal model, mice were injected with MPTP 25 mg/kg combined with probenecid (250 mg/kg) every 3.5 days, 10 times in total. Due to the different doses, routines and durations of the treatments with MPTP/MPP^+, different results were obtained.

In the cellular model of PD, mitochondrial dehydrogenase activity was decreased by inducing oxidative stress. The production of the oxidants ROS and RNS was enhanced, with increased ROS production triggered by the higher expressions of NADPH-oxidase. Antioxidant concentrations and mRNA expressions of the antioxidant enzymes in the cellular model were found to be activated, which may reflect a defense in cells against the effect of MPTP/MPP^+ in the PD model. Increased expressions of pro-oxidant and antioxidant enzymes, along with increased antioxidant levels, showed that the oxidative system and anti-oxidative system were both activated in the cellular PD model. In the acute animal models, there was no change in the oxidative system or the anti-oxidative system. In the midbrain of the chronic animal model, mRNA expressions of the pro-oxidant enzyme NADPH-oxidase and the antioxidant enzyme catalase were found to be suppressed.

The differences from these three models may result in part from different MPTP/MPP^+ administrations. In the chronic model, mice were injected with MPTP, which can indirectly

affect the dopaminergic neurons in this model through different DA pathways. This indicates that many uncertain complicated factors in the body system may affect the results. The dopaminergic neuron-like cells in the cellular model and the striatal slice tissues were submerged in higher concentrations of MPP^+ for a longer incubation period, and lower concentrations of MPP^+ for a shorter incubation period, which could also cause different results. In addition to differences in MPTP/MPP^+ administration, the presence or absence of glial cells may have affected the difference in results. The cellular study only contained neurons, while the animal models contained both neurons and glial cells. Glial cells, including microglia and astrocytes, are non-neuronal cells which provide support and nutrition, maintain homeostasis, and participate in signal transmission in the CNS (Vernadakis, 1996). Glial cells normally play neuroprotective roles. However, in response to adverse stimuli, they may contribute to neuronal damage. Recent studies have demonstrated that acute microglial activation can protect neurons from acute brain injury (reviewed by Suffredini *et al.*, 1999), but chronic microglial activation may induce neurodegeneration, as they can also produce a large amount of O_2^- and other neurotoxins (Hald *et al.*, 2007). Different from microglia, astrocytes can secrete a number of neurotrophic factors, which have been reported to protect midbrain dopaminergic neurons from MPP^+-induced neurotoxicity (Zhang *et al.*, 2006). In the cellular model of PD, glial neuroprotection was absent, and the only defending function available to cells was the enhancement of antioxidant functions. Thus, when oxidants were increased in the cellular model, anti-oxidative systems were also increased in response. This may also be the reason that, in the present study, the acute brain slice model showed no changes to the oxidative system, as the presence of glial cells may provide protection against the acute effects of

MPTP/MPP^+. However, in the midbrain of the chronic model, both pro-oxidant and antioxidant enzyme amounts were decreased. As mentioned above, neurodegeneration may be caused by over-activated microglial cells in response to chronic MPTP/MPP^+ insult. Therefore, as the results show, active microglia might have caused cell damage and have destroyed both pro-oxidant and antioxidant enzymes after chronic MPTP/MPP^+ treatment. Another factor in the differing results could be that mRNA expressions might not completely reflect enzyme functions. Both protein expressions and the activities of pro-oxidant enzymes and antioxidant enzymes require further investigation.

Another aim of the present study was to explore the relationship between changes in the fatty acid profile and oxidative stress. MPTP with different doses, routines and durations also differently affected fatty acid concentrations in the two animal models. In the acute model, the biosynthesis of the n-6 fatty acids LA and AA was enhanced by acute MPTP administration. However, in the chronic animal model, the concentrations of fatty acids were remained unaffected by MPTP. The reason for these different results is presumably the same as discussed in Chapter 3: that under the chronic effect of MPTP, the increased n-6 fatty acids AA and LA have already been metabolized into PGE2 and other inflammatory mediators, reducing the increase in fatty acids observed in the acute model of PD. Another possibility is that increased n-6 fatty acids may be induced by rapidly increasing cellular stress, as seen in acute MPTP/MPP^+ treatment, but not in chronic treatment, which can trigger enzymes involved in fatty acid biosynthesis, including sterol-regulatory element binding protein (SREBP) (Colgan, 2007).

Together, though MPTP-induced changes did not appear in the pro-oxidant and antioxidant enzymes in the acute animal study, the fatty acid profile was changed. This result may indicate that there is no direct relationship between MPTP-induced changes in fatty acids and oxidative stress. The results from the chronic study further support this finding, because while the oxidative and anti-oxidative systems were both suppressed by MPTP, there was no change found in the fatty acid profile. This suggests that the fatty acid profile and pro-oxidant and antioxidant enzymes may be affected by MPTP via two independent pathways.

4.3.2 Changes induced by EPA alone in the oxidative and anti-oxidative systems, and the fatty acid profile in the different models

In the three models, EPA was also treated with different doses, routines and durations. In the cellular model, differentiated SH-SY5Y cells were submerged for 48 to 96 hours in a culture medium containing EPA at a concentration of 100 μM. In the acute animal model, a diet with 0.8% EPA was given to experimental mice for 6 weeks, while in the chronic animal model, and 0.8% EPA diet was given to experimental mice for 12 weeks in total. Although different treatments with EPA were carried out, EPA did not affect the oxidative and anti-oxidative systems in any of these three models. Firstly, in the cellular model, no EPA-induced change in the oxidative and anti-oxidative systems was found other than a decrease in the pro-oxidant enzyme nNOS, which did not induce any change in RNS production. Secondly, in the acute animal model, no change was found in the oxidative or anti-oxidative systems. Finally, in the midbrain and the striatum of the chronic animal model, EPA had no effect on the oxidative and anti-oxidative systems either. Taken

together, it was found that, based on the direct effects of EPA on cells, in mice fed with EPA for 6 weeks in the acute study and in mice fed EPA for 12 weeks in the chronic study, EPA did not induce any effects on the oxidative and anti-oxidative systems under normal conditions (without MPTP/MPP^+ insult). However, in the cellular model, EPA did increase mitochondrial dehydrogenase activity, though there was no association with any EPA-induced change in the oxidative and anti-oxidative systems. This may indicate that EPA increases mitochondrial dehydrogenase activity by other mechanisms, such as via anti-inflammatory or anti-apoptosis pathways. These mechanisms have been reported by both our team and other scientists, which demonstrated that EPA may improve depression via its anti-inflammation function such as decreasing IL-1beta and PGE2. EPA may exert beneficial effects on cell survival by modulating neurotrophin receptor expressions, such as brain-derived neurotrophic factor (Song *et al.,* 2009; Kou *et al.,* 2008).

With respect to the fatty acid profile, the EPA intakes for both 6 weeks and 12 weeks can induce similar increases in EPA and DPA concentrations in the brain tissue. This means that dietary EPA can be successfully transformed into the n-3 fatty acids EPA and DPA in the brain in 6 weeks or more.

4.3.3 The effect of EPA on MPTP/MPP^+-induced changes in the oxidative and anti-oxidative systems, and the fatty acid profile in the different models

In the three models, EPA was used to treat MPTP/MPP^+-induced changes. In the cellular model, treatments of MPP^+ at 50 μM and100 μM and EPA at 100 μM were administered to SH-SY5Y cells. In the acute animal model, mice were fed EPA for 6 weeks and their brain

slices were treated with MPP^+ for 4 hours after the animals were sacrificed. In the chronic animal model, animals were fed with EPA for 12 weeks, and from the seventh week onwards they were injected with MPTP for a total of 10 times, with 3.5 days between each injection. Due to the different doses, routines and durations of the treatments with MPTP/MPP^+ and EPA, different results were obtained. In the cellular PD model, EPA increased mitochondrial dehydrogenase activity by attenuating the over-produced oxidants ROS and RNS, since EPA could down-regulate ROS production by suppressing NADPH-oxidase. However, EPA had no effect on the oxidative and anti-oxidative systems in the other two animal models. The possible reasons for the differences are discussed. Firstly, MPTP/MPP^+ did not significantly change mRNA expressions of these enzymes in the acute animal model, and then making EPA effects indistinguishable from baseline. Secondly, as mentioned in the discussion of the chronic model in Chapter 3, the EPA feeding duration may not be sufficient to attenuate the effect of MPTP/MPP^+ on the oxidative and anti-oxidative systems, since EPA had a potential attenuating effect on antioxidant enzymes such as catalase. If the treatment had lasted longer, the effect could have shown increased significance. Thirdly, the mRNA expression used in the present study may not have been enough to reveal enzyme functions, so protein expressions and enzyme activities may still need to be tested in the future. Also, the MPTP dose chosen in the chronic study may have been too high for EPA to attenuate the effect.

For the effect of EPA and MPTP/MPP^+ treatments on the fatty acid profile, EPA intake attenuated the increased concentrations of the n-6 fatty acids AA and LA in the acute animal study, which may have reduced the risk of pro-inflammatory triggers and also

confirmed that n-3 fatty acids can compete with n-6 fatty acids with respect to incorporation into cell membrane phospholipids (Lands, 1992). In the acute and chronic models, MPTP administration did not change the EPA-induced increases in both the n-3 fatty acids EPA and DPA. As hypothesized, if EPA significantly increased n-3 fatty acid concentrations under MPTP/MPP^+ insult, EPA should have reduced the MPTP/MPP^+-induced changes in the oxidative and anti-oxidative systems in the chronic model. However, this hypothesis was not proved in the present study. A longer EPA treatment may be required in future studies.

In summary, it was found that MPTP/MPP^+ is more sensitive in the cellular model than the other two models for testing the mechanism of oxidative stress. The results from the present studies also suggest that the cellular model is the most suitable model for testing new treatments, such as EPA, for PD. Here, several reasons should be emphasized: 1) It is an economical model for initial studies, and can be used for long term research; 2) Because the model created with human neuron-like cells, direct oxidative stress effects on the neuronal model can be evaluated and 3) Dopaminergic neuron-like cells are more sensitive to MPTP/MPP^+, which has been proven again by this study. From these three experiments, changes in oxidative and anti-oxidative systems in the animal models were not as significant as those observed in the cellular model. The reasons include many as-yet-unexplored factors, including the protective effect of glial cells and interactions between neurotransmitters and inflammation, which are involved in the *in vivo* models after MPTP/MPP^+ administrations. Additionally, the tissue amount was insufficient and thus

results could not readily reach significance in the present study. These methodological aspects are also essential and should be addressed in future studies.

4.3.4 Evaluation of this study

As mentioned, oxidative stress plays an important role in the pathogenesis of PD, and to understand the mechanism of oxidative stress was one of the major aims of this study (reviewed by Jenner *et al.*, 1992). It is known that oxidative stress may result from the imbalance between the oxidative system and the anti-oxidative system (reviewed by Mena *et al.*, 2008); whereas the interrelation between these two systems in PD is still unclear. This study was the first to show the interrelation between the oxidative system and the anti-oxidative system in PD models, as expressed in cellular mechanisms and both acute and chronic systematic mechanisms, which can help us to understand the role of oxidative stress in PD and then discover suitable therapies. Current therapies are unable to help patients in the late stage of PD and may cause severe side-effects. This study provided the novel finding that EPA, as an antioxidant, could attenuate oxidative stress in the cellular model induced by MPP^+ and has the potential to benefit the function of antioxidants in the chronic PD model. Therefore, this study may provide a better understanding of the pathogenesis of PD and supply evidence for the beneficial effects of EPA therapy for PD patients.

4.4 Future research

Although some evidence has been obtained from this project to help us to understand oxidative mechanism of PD and evaluate the efficacy of EPA as a new therapy, some

questions still require further investigation. First, although differentiated SH-SY5Y cells are a suitable model for PD research at the cellular level, they exist in an *in vitro* environment without microglia and astrocytes. Glial cells provide support and nutrition to neurons, maintain homeostasis, and play an important role in signal transmission in the nervous system. Microglia also form the first and main line of active immune defense in the CNS (reviewed by Male *et al.*, 2006). As microglia and astrocytes are essentially involved in PD, and could be activated by MPTP/MPP^+ to protect neurons at the acute stage (Male *et al.*, 2006; Mount *et al.*, 2007), co-cultured SH-SY5Y cells and microglial cells have already been studied at our lab. Co-culturing SH-SY5Y cells and glial cells can act as a better model, which is closer to the natural environment of the human body. This cellular model will be used to research oxidative stress mechanisms in the future.

Second, the present study showed that EPA treatment without MPTP/MPP^+ insult can only enhance mitochondrial dehydrogenase activity, but that it cannot affect the oxidative and anti-oxidative systems. Therefore, EPA may increase mitochondrial dehydrogenase activity via other mechanisms. For example, it has recently been reported that EPA can up-regulate the expressions of neurotrophins and their receptors, and that EPA can inhibit inflammatory responses (Kou *et al.*, 2008; Song *et al.*, 2009). Thus, other mechanisms by which EPA can affect MPTP/MPP^+ could be future directions of study.

Thirdly, the cellular model showed that EPA can reduce RNS production, but not through its enzyme nNOS. It is possible that RNS may be partly produced by a non-enzyme-dependent pathway. This needs to be further investigated in the future to clarify the

pathway by which EPA lowers RNS production. Furthermore, in Chapter 3 it is suggested that the EPA feeding time may need to be extended due to some data that only approached the significance. Future study should be conducted with a longer EPA feeding time, which may improve the efficacy of EPA on the MPTP animal model.

It was also found that the low amount of brain tissues in the quantitative PCR experiment produced variable results and induced a high possibility of producing non-significant data. To solve this problem, more animals would be needed for this oxidative stress mechanism study in PD, which could provide a clearer picture regarding the efficacy of EPA on the effect of MPTP/MPP^+ through the oxidative stress pathway. In addition, GC methods should be further developed to detect more fatty acids in the brain. The total amount of n-3 fatty acids, n-6 fatty acids and the ratio of n-3 to n-6 fatty acids in the mouse brain would then be more accurately reported in later research. And last but not least, other than NADPH-oxidase being measured by quantitative PCR and Western blot, other pro-oxidant enzymes and antioxidant enzymes were only measured by PCR at the mRNA level. However, results involving mRNA expressions cannot completely reflect enzyme function. Thus, in future studies, enzyme protein expressions or activities should be further investigated to give more evidence to support the mechanisms of oxidative stress and the effects of EPA.

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Appendix

Melting curve analysis has been always performed at the end of each PCR assay to control for specificity; specific reactions should result in a single melting peak corresponding to the PCR product being amplified. In this study, each reaction has shown the high specificity (as indicated the single melting peak in melting curve analysis) (Figure A.1, A.2, A.3, A.4).

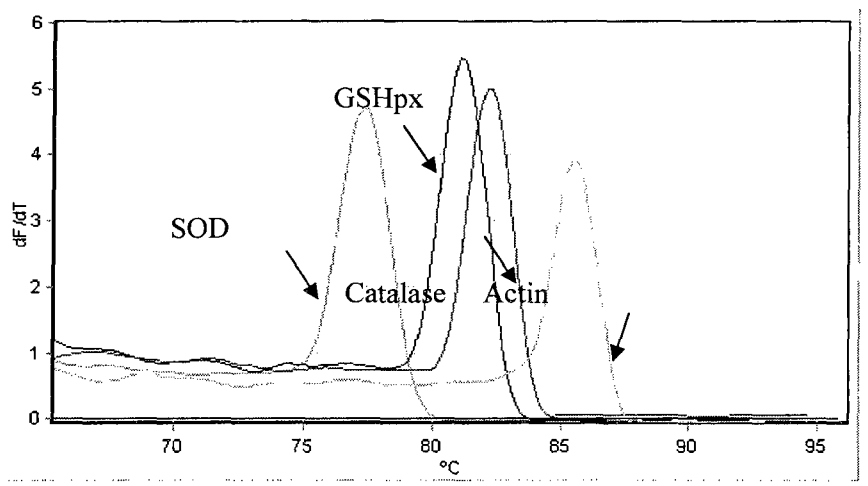

Figure A.1. The melting curves of antioxidant enzymes (human)

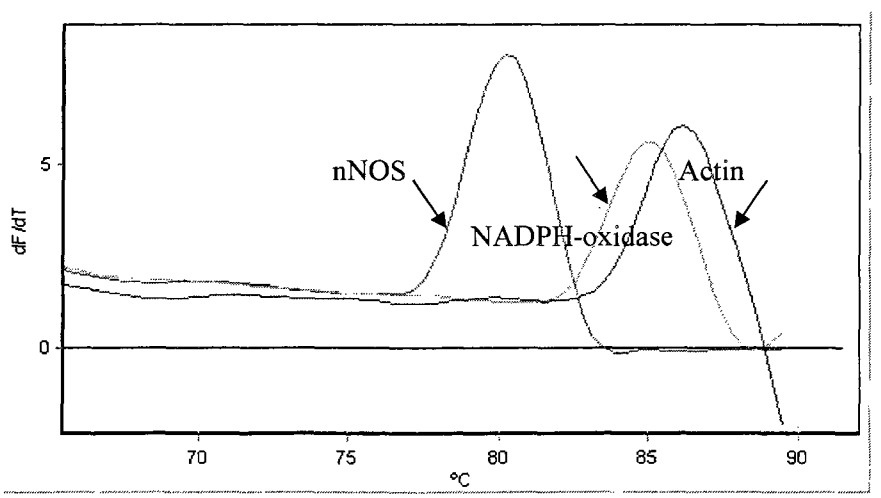

Figure A.2. The melting curves of pro-oxidant enzymes (human)

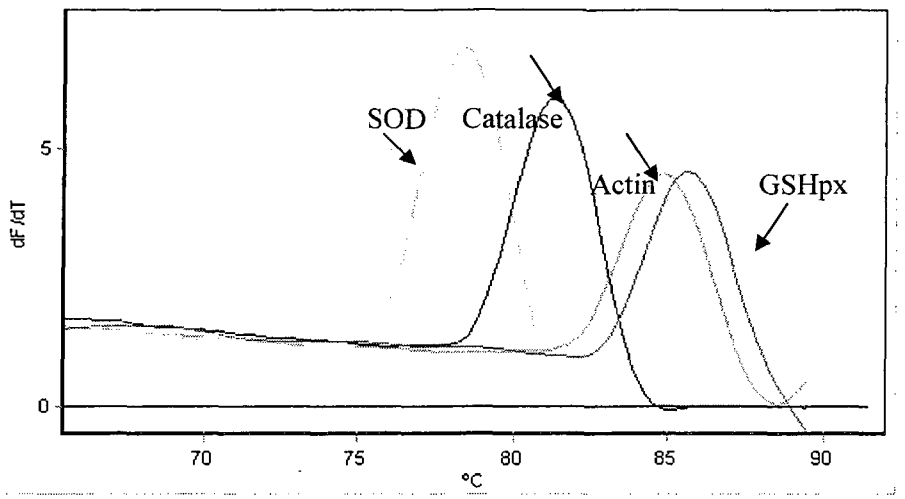

Figure A.3. The melting curves of antioxidant enzymes (mouse)

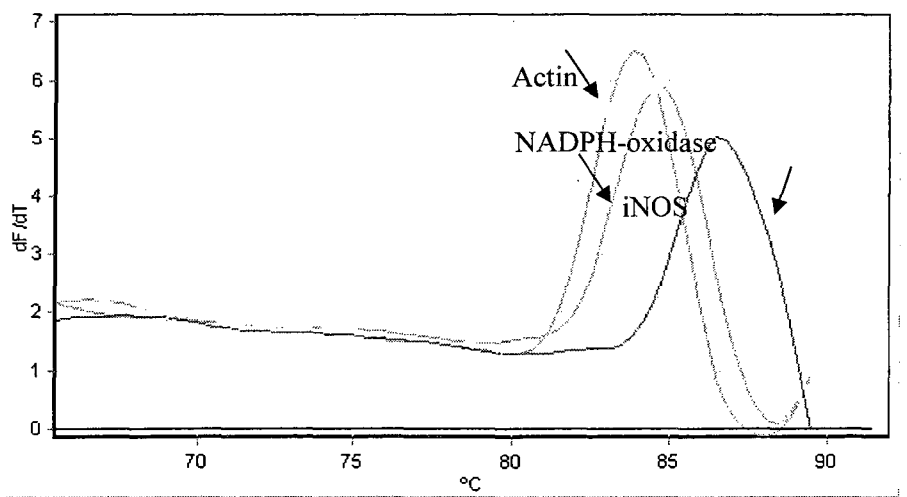

Figure A.4. The melting curves of pro-oxidant enzymes (mouse)