THE ROLE OF CYTOSOLIC PHOSPHOLIPASE A2 IN MICROGLIAL SIGNALING PATHWAYS DURING NEUROINFLAMMATION

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the [thesis or dissertation] entitled

THE ROLE OF CYTOSOLIC PHOSPHOLIPASE A2 IN MICROGLIAL SIGNALING PATHWAYS DURING NEUROINFLAMMATION

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DEDICATION

I wish to dedicate this work to my grandfather. He had battle with Alzheimer's disease for 14 long years and passed away earlier this year, in January, in his home country of Taiwan. He had always been and will continue to be the inspiration for my pursuit of knowledge in neuroscience and my passion for scientific research.

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta
AA	Arachidonic acid
AACOCF3	Arachidonyl trifluoromethyl ketone
AD	Alzheimer's disease
ANOVA	Analysis of variance
BEL	Bromoenol lactone
C3G	Cyanidin-3-O-glucoside
CM-H2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
CNS	Central nervous system
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipase A2
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Elderberry
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
g	Gram

GAFP	Glial fibrillary acidic protein
GFP	Green fluorescence protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hour
H2O2	Hydrogen peroxide
IFNγ	Interferon gamma
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
iPLA2	Calcium-dependent phospholipase A2
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAFP	Methylarachidonyl-fluorophosphonate
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minutes
mL	Milliliter
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
NaNO2	Sodium nitrate

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NO	Nitric oxide
O2•-	Superoxide
OH•	Hydroxyl radicals
ONOO-	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered solution
PD	Parkinson's disease
PGE2	Prostaglandin E2
PLA2	Phospholipase A2
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Standard error of mean
SDS	Sodium dodecyl sulfate
SF	*Sutherlandia frutescens*
sPLA2	Secretory phospholipase A2
STAT	Signal tranducer and activator of transcription
TBS	Tris-buffered solution
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor α
wks	Weeks

µg	Microgram
µL	Microliter
µM	Micromolar
ºC	Celsius

THE ROLE OF CYTOSOLIC PHOSPHOLIPASE A2 IN MICROGLIAL SIGNALING PATHWAYS DURING NEUROINFLAMMATION

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ABSTRACT

Oxidative and nitrosative stress is known to play an important role in neurodegenerative and neuroinflammatory diseases, such as Alzheimer's disease, Parkinson's disease, ischemic/hemorrhagic stroke, and traumatic brain injury. Microglia are the primary innate immune cells in the central nervous system (CNS). These cells are the first line of defense against foreign pathogens, and the exhibit multiple physiological roles including removing cellular debris and maintaining tissue homeostasis. Pathologic activation of microglia, which is frequently secondary to neurodegenerative or neuroinflammatory processes, can lead to the release of proinflammatory cytokines, reactive oxygen species (ROS), reactive nitrogenous species (RNS), excitotoxic neurotransmitters, metalloproteases, and other pro-inflammatory/cytotoxic factors. Propagation of inflammation in neurodegenerative conditions and can be a contributor of neuronal cell death.

Cytosolic phospholipase A2 (cPLA2) belongs to a family of phospholipases that act as the principle producers of arachidonic acid (AA) in cells. During the process of inflammation, AA released from phospholipids is metabolized by cyclooxygenase-1/2 (COX1/2) and lipoxygenases, and in turn can be converted to prostaglandins, prostacyclin and thromboxane. This is an important pathway of inflammation, with COX1/2 being the popular target for non-steroidal anti-inflammatory drugs (NSAIDs). The role of cPLA2 and its downstream pathway has not been investigated in depth in microglial cells during CNS neuroinflammation. Using primary microglial culture prepared from cPLA2 knockout mice, we found that oxidative and nitrosative stress responses were significantly attenuated in cPLA2 knockout mice as compared with wildtype control. The same phenomenon was observed using pharmacological inhibition and siRNA knockdown of cPLA2 in BV2 microglial cells. Interestingly, unlike macrophages, inhibition of COX1/2 did not result in significant decrease in oxidative/nitrosative responses in BV-2 cells. Instead, lipoxygenase (LOX) inhibition, or more specifically LOX-12 and LOX-15, could significantly suppress ROS/NO production in BV2 cells.

Plants form the basis of traditional medicine in different civilizations throughout thousands of years. The use of herbal medicine was based on centuries of anecdotes and practitioners' experience. Recent incorporation of modern research methods to traditional medicinal investigation has shed light on the mechanism of action for many of these herbal products. Many medicinal plants and extracted compounds have been

hypothesized or shown to ameliorate neurological conditions. In order to better understand the mechanism of action of botanical compounds on microglial activation pathway, an experiment was carried out in which mice were fed with control diet, elderberry diet and Sutherlandia diet for two months and followed by global cerebral ischemia by occlusion of the bilateral common carotid arteries. Mice fed with either elderberry or Sutherlandia diet demonstrated significant less motor deficits (by rotarod test). Histologic staining by cresyl violet stain showed significant decrease in neuronal cell death and decrease in microglia activation by either diet group. Immunohistochemical staining further showed colocalization of phospho-ERK and p47phox to microglial cells and decrease expression of the oxidative proteins in mice fed with either botanical diets.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Neurological diseases and neuroinflammation

With the elderly population continues to rise in the United States, due to medical advancements and improved prevention of diseases, the prevalence of neurological diseases has also risen dramatically in this aging population. Traditional metric assessments with clinical measurements, such as mortality, often underestimate the global health burden from neurologic diseases. The World Health Organization (WHO) published an official report in 2005 using a new metric assessment termed "disability adjusted life year" (DALY), which incorporated early mortality and morbidity of nonfatal chronic diseases in the assessment of health status. This assessment showed that neurological disorders lead the percentage of global health burden when compared to the causes of all other diseases, including cardiovascular diseases, malignancy, HIV, malaria, tuberculosis…etc. More importantly, the prevalence of these disorders is projected to increase, with cerebral vascular diseases and Alzheimer's disease as the leading contributors to this index.

Neuroinflammation, defined as inflammation in the central nervous system (CNS), plays an important role in the pathophysiology of numerous neurological disorders. Examples include neurodegenerative disease, such as Alzheimer's disease and Parkinson's disease; neurovascular disorders, such as ischemic or hemorrhagic stroke;

neuro-infectious causes, such as meningitis, encephalitis and HIV-associated conditions; neuro-immune conditions, such as multiple sclerosis and neurosarcoidosis; and neuro-trauma, such as traumatic brain or spinal cord injury. The mechanisms underlying these disorders vary depending on acute or chronic status of the disease and the different cell types involved in the progression of the disease. Among the immune active cells in CNS, microglia are without a doubt the most important in the disease pathology, regardless of being the causative source of neuronal damage or the propagator of disease progression. In recent years, there is a special emphasis on the impact of microglia on the pathophysiology of stroke.

Stroke

Stroke is the second leading cause of death world-wide after ischemic heart disease, and is the primary cause of acquired disability in the United States (Davis and Donnan 2012). Stroke is characterized by focal neurologic deficits caused by alterations in circulation that supplies the encephalon. Based on the etiology, stroke is divided into two major types, namely ischemic and hemorrhagic stroke. Ischemic stroke results from a decrease of blood flow to the brain due to formation of a thrombus, blockage from emboli, cerebral hypoperfusion…etc. It accounts for 80% of all cases of stroke. On the other hand, hemorrhagic stroke results from the accumulation of blood in the brain tissue due to rupture of blood vessels in the brain, and accounts for 20% of all cases of stroke (van der Worp and van Gijn 2007, Davis and Donnan 2012). Although the mortality of stroke has decreased in developed countries due to early recognition of symptoms and accelerated intervention, roughly half of the stroke survivors are still left with permanent

neurologic deficits, resulting in some degrees of physical or cognitive impairment (Di Carlo 2009). One can imagine the economic burden imposed by this disease resulting from the acute medical care, long-term rehabilitation, and costly efforts to integrate disabled patients into society. An international study estimated that, on average, 0.27% of gross domestic product was spent on stroke by national health systems, and stroke care accounted for about 3% of total health care expenditures (Di Carlo 2009).

Current acute therapies for stroke in human patients

Current acute treatment of stroke primarily focuses on the process of recanalization of affected blood vessels, which relies on two methods: (1) the breakdown of clots (thrombolysis) and (2) mechanical retrieval of clots (thrombectomy). Despite extensive research effort made in the past few decades, recombinant tissue plasminogen activator (rtPA) remains the only FDA-approved non-invasive therapy (Yarbrough, Ong et al. 2015). Physiologically, tPA (along with plasmin) are the enzyme that cleavages the plasminogen, a zymogen, into the functionally active serine protease plasmin, which is responsible for fibrinolysis of thrombi and emboli. The administration of rtPA aims to increase the overall enzymatic of this process, and thereby facilitate the breaking down of fibrin-rich thrombus and clots that are responsible for causing stroke (Ouriel 2004). Unfortunately, even in developed Western countries, only a small portion of patients with ischemic stroke can receive intravenous rtPA. Reasons are mainly due to restriction from the short therapeutic window within 3-4.5 hours after onset (or more objectively, last-seen-normal) (Group. 1995), as well as contraindications such as recent surgery, history of intracranial hemorrhage (ICH), and late presentation (de Los Rios la Rosa, Khoury et

al. 2012, Jauch, Saver et al. 2013). Even if the patients meet the criteria and receive the thrombolysis therapy, there is an inherent risk of hemorrhagic transformation which positively correlates with the initial ischemic infarct volume. Furthermore, while recanalization rate positively correlates with functional outcome of patients, recanalization rate with IV-rtPA have been reported at merely 17-38% in the literature (van der Worp and van Gijn 2007).

Recent advancements in mechanical thrombectomy technologies and techniques have demonstrated efficacy in improving ischemic stroke outcome. The Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands (MR CLEAN), published in the New England Journal of Medicine in early 2015, was the first to show definite support favoring the use of thrombectomy (Berkhemer, Fransen et al. 2015). Immediately following the publication of MR CLEAN, several other studies were terminated early, due to overwhelming evidence of outcome favoring the group undergoing thrombectomy. These include trials such as the Endovascular Revascularization with Solitaire Device Versus Best Medical Therapy in Anterior Circulation Stroke Within 8 Hours (REVASCAT), Evaluation Study of Congestive Heart Failure and Pulmonary Artery Catheterization Effectiveness (ESCAPE), and Extending the Time for Thrombolysis in Emergency Neurological Deficits–Intra-Arterial (EXTEND-IA) (Chen, Ding et al. 2015, Yarbrough, Ong et al. 2015). While the newly published evidence may seem encouraging, limitations still exist in these clinical trials. One such example is the possibility of selection bias, as patients were prescreened and selected based on likelihood of success prior to thrombectomy

procedure. Therefore, similar to the administration of rtPA, only limited number of patients may benefit from mechanical recanalization.

In vivo stroke models

Many animal models have been proposed and developed for stroke. While mice and rats are the most commonly used species for the investigation of stroke, larger animals have been made available, including rabbits and non-human primates (Casals, Pieri et al. 2011). Due to the complexity and variability of cerebral vasculature among species, variations in tissue damage response from differences in age, and distributions of infarcted areas based on stroke induction techniques, it is important for researchers to select appropriate models based on the experimental design. In general, there are 3 major types of animal models for stroke. The most commonly used stroke model is characterized by occlusion of specific cerebral arteries by ligation or thrombosis. The areas of infarct with this method are more well-defined, with more subcortical necrosis, followed by apoptosis of neurons in the surrounding penumbra. A second way to induce stroke is by injection of preformed emboli into the cerebral vasculature. Due to the variability of size and instability of the clot, the infarct area is less predictable and tend to be concentrated in the cortical areas. This is due to smaller size and better ability of clots to penetrate into smaller vessels. Unlike the previous two categories where stroke is induced, genetic models are sometimes used to investigate spontaneous development of stroke. Examples of these include the hypertensive model or Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) models. Since stroke develops spontaneously from higher intrinsic risk factors, this

model is more suitable for aging animals and not as controlled as the induced methods (Casals, Pieri et al. 2011).

The global cerebral ischemia model, induced by occlusion of the bilateral common carotid arteries (BCCAO) has been successfully carried out using the Mongolian gerbils because 80% of these animals lack communicative arteries, and thus preventing collateral blood flow from the posterior circulation to the ischemic regions (Wang, Xu et al. 2002, Wang, Sun et al. 2005, Wang, Sun et al. 2009). Subsequent studies demonstrated the ability to apply this technique with small animals, such as the c57bl6 mice (Chuang, Cui et al. 2014). Animals that have undergone BCCAO are prone to developing ischemic damage in the hippocampal and striatal regions (Lin, Cao et al. 2000, Wang, Sun et al. 2005, Yoshioka, Niizuma et al. 2011).

Several methods have evolved for the focal cerebral ischemia model. (1) Professor Chung Y. Hsu created a surgical model for transient ligation of the middle cerebral artery (MCA) of rat as a model that closely mimics the ischemic phenomena in human patients with focal MCA stroke. Areas most affected by the MCAO model are situated at the neocortex and peri-capsular regions, and animals typically exhibit contralateral sensory-motor deficits (Lin, He et al. 1993, Schallert, Woodlee et al. 2003, Lin, Wang et al. 2004). (2) Subsequently, a method in which filament is inserted to block the MCA in rat and mice was develop to simulate the effect of a transient embolic clot (Gu, Kaul et al. 2002, Gu, Cui et al. 2005). (3) More recently, Dr. Zezong Gu's group have developed a protocol to recreate the same phenomenon in which a fibrin-rich

embolus is made and injected into the MCA (Cui, Chen et al. 2012). (4) The photothrombotic model, on the other hand, offers a minimally invasive approach to inducing stroke. Briefly, the blockage is induced first by injection of photosensitive dye (Rose Bengal), which is activated by illumination of a cold light source. The activated dye is able to cause endothelial damage, with platelet activation and thrombus formation (Labat-gest and Tomasi 2013, Li, Zhang et al. 2014). Unlike the MCA stroke, which produce large areas of infarct, the photothrombolic model produces small infarction in the brain with confined pathology and functional deficits.

Cellular and molecular events in stroke

It is important to recognize that while the etiologies of stroke differ from case to case, the mechanism of neuronal cell death may be similar. In most cases, tissue hypoxia and glutamatergic excitotoxicity are involved in the induction of neuronal cell death.

The mechanisms linking hypoxia to neuronal cell death are complex, and extensive studies have focused on the underlying mechanisms of oxidative stress and inflammatory responses following ischemia/reperfusion (I/R) (Chen, Kim et al. 2011, Chen, Yoshioka et al. 2011). On a cellular level, studies have demonstrated the role of NADPH oxidase and activation of the mitogen-activated protein kinase (MAPK) pathways in production of reactive oxygen species (ROS), and signaling events leading to mitochondrial dysfunction and activation of apoptotic pathways in neurons, glia and endothelial cells (Chen, Kim et al. 2011, Yoshioka, Niizuma et al. 2011). Studies have also shown that cytosolic phospholipase A2 (cPLA2), the principle enzyme responsible

for the generation of arachidonic acid, is upregulated in experimental stroke, and further induces the expression of cyclo-oxygenase-2 (COX-2) and production of prostaglandin E2 (PGE2). Inhibition of cPLA2 with pharmacological inhibitors could effectively protect against focal ischemic damage in mice after ischemia/reperfusion (Kishimoto, Li et al. 2010, Zhang, Barasch et al. 2012).

Recently, there is increased interest to search and screen for compounds, both pharmacological and nutraceutical, that may ameliorate ischemic damage by up-regulating the anti-oxidative Nrf2 pathway and enhancing the synthesis of heme-oxygenase-1 (HO-1). A study by Chao et al. with transgenic mice, showed that overexpression of HO-1 can attenuate brain damage after ischemia/reperfusion (Chao, Ma et al. 2013). Another study by Chen et al. attributed the protective effect of octreotide against experimental stroke to upregulation of Nrf2 and HO-1, as well as suppression of NF-κB expression (Chen, Wang et al. 2012). Studies by others have also demonstrated beneficial effects on stroke from naturally occurring phenolic compounds. A few examples of these compounds include Lycium barbarum polysaccharides from goji berries (He, Pan et al. 2014), tetramethylpyrazine from natto (Kao, Chang et al. 2013), genistein from fava beans/soybeans (Wang, Tu et al. 2013), ursolic acid from apple peels (Li, Zhang et al. 2013), and Notoginsenoside R1 from tienchi ginseng (Meng, Wang et al. 2014).

Most studies, as discussed above, have primarily focused on arresting the process of neuronal necrosis and apoptosis secondary to ischemia/reperfusion and tissue hypoxia.

However, with improved understanding of the function and interactions of glia and other cell types in the brain, there is increasing interest to develop novel strategies to manipulate the behavior of glia to ameliorate disease pathology in *in vivo* models of experimental stroke. Examples include effort to manipulate expression of M1 vs M2 microglia phenotypes at specific time after the onset of stroke in order to limit the effects of oxidative and inflammatory stress, as well as promoting tissues repair (Taylor and Sansing 2013, Pan, Jin et al. 2015, Suenaga, Hu et al. 2015, Tang and Le 2015, Xia, Zhang et al. 2015). This specific mechanism will be discussed in further detail in the following sections. While these strategies have not been applied clinically, the results based on cellular and animal studies appear promising.

Microglia

Microglia physiology

Microglia, the resident immune cell in the central nervous system, were initially described by Franz Nissl and Ford Robertson in late 19th century by histology and light microscopy techniques (Nissl 1899, Robertson 1900). The ability of microglial cells to undergo morphological change into ramified microglia was described by Victor Babeş while studying a case of rabies (Babeş 1892). However, it was actually Pio del Rio-Hortega, student of Ramón y Cajal, who officially coined the term microglia, performed extensive research on these cells, and thus was considered the "father of microglia" (del Rio-Hortega and Penfield 1892, del Rio-Hortega 1932). Due to the limitation of identification methods, and relying mainly on silver carbonate staining, del Rio-Hortega hypothesized that microglia originated from invasion of mesodermal pial elements into

the nervous system, although he also speculated about blood mononuclear cells as another potential source (Tambuyzer, Ponsaerts et al. 2009). The debate on microglial ontogeny went on for decades, with more favoring the neuroectodemal as compared to the myeloid-monocytic hypothesis. At the same time, knowledge of microglia was limited and stagnant for most of the 20th century; a majority recognizing them as cells that populate as a reactive process to injuries, and possess functions similar to macrophages, such as the ability to migrate, populate and perform phagocytosis.

With the advancement in labeling and imaging techniques, our understanding of microglia today has dramatically expanded. Current consensus in knowledge suggests that these cells derive mainly from the primitive myeloid cells produced in the yolk sac during embryonic development, and subsequently migrate to the CNS during the early embryonic stage before maturation of the hematopoietic system in the bone marrow (Ginhoux, Greter et al. 2010, Schulz, Gomez Perdiguero et al. 2012, Yona, Kim et al. 2013). Microglial cells account for roughly 10-15% of total cells in the CNS, and can be found in almost all parts of the adult brain and spinal cord (Lawson, Perry et al. 1990).

As immune competent cells, microglia are capable of performing immune surveillance in the CNS. They act as the first line of defense in the CNS against infections and react to neuronal insults. In the presence of stimuli, activated microglia exhibit multiple functions such as phagocytosis of exogenous pathogens, scavenging apoptotic cellular debris, and to some extent, act as antigen presenting cells.

Recent evidence suggests that microglia actively participate in synaptic pruning during neurodevelopment (Tremblay, Stevens et al. 2011). For example, process-bearing activated microglial cells are frequently associated with dendritic spines in an experience-dependent manner, and perform active roles in remodeling synaptic circuits (Wake, Moorhouse et al. 2009, Tremblay, Lowery et al. 2010). Mice deficient in CX3CR1, a microglia-specific chemokine receptor that binds to CX3CL1 on neurons, show impaired synapse development (Harrison, Jiang et al. 1998, Jung, Aliberti et al. 2000). Similarly, studies have demonstrated the ability of microglia to engulf and eliminate extranumerary synapses with the help of the complement system (Stevens, Allen et al. 2007).

Studies to investigating glial interaction with neurons and other cells in the CNS have unveiled important functions of microglia in the regulation of synaptic transmission in neurons. Microglia are closely associated with neurons and astrocytes in the brain. Studies using mouse hippocampal slices demonstrated that microglia activation led to increase in neuronal excitatory postsynaptic potential via the AMPA receptor and through the release of ATP, activation of P2Y1 receptor on astrocytes, and subsequent astrocytic glutamate release (Pascual, Ben Achour et al. 2012).

Microglia in neuroinflammation: the M1 vs. M2 hypothesis

While recent advances in microglial research had shed light on the physiological functions of these cells, more studies have focused on unveiling mechanisms of their inflammatory functions, particularly under pathological conditions. In many neurodegenerative diseases, microglial cells become reactive and readily populate the

sites of lesions. Similar to macrophages, microglia can also transform into M1 and M2 phenotypes with polarizing properties. The M1 phenotype favors the classical activation and inflammation induced by cytokines and endotoxins, whereas M2 microglia release anti-inflammatory factors that are neuroprotective during the reparative stage (Fig. 1.1) (Franco and Fernandez-Suarez 2015, Hu, Leak et al. 2015, Tang and Le 2015). With a better understanding of when each phenotype predominates during disease pathology, studies have begun to focus on ways to limit M1 activation and stimulate M2 transformation. Successful development of this modulation is important in ameliorating outcomes of neurological diseases, such as experimental stroke and traumatic brain injury (Hu, Li et al. 2012, Wang, Zhang et al. 2013, Pan, Jin et al. 2015, Wang, Shi et al. 2015, Xia, Zhang et al. 2015). Therefore, it is of paramount importance that more studies are done to understand mechanism(s) on the signaling transduction pathways and enzymes associated with each phenotype with regard to the disease conditions.

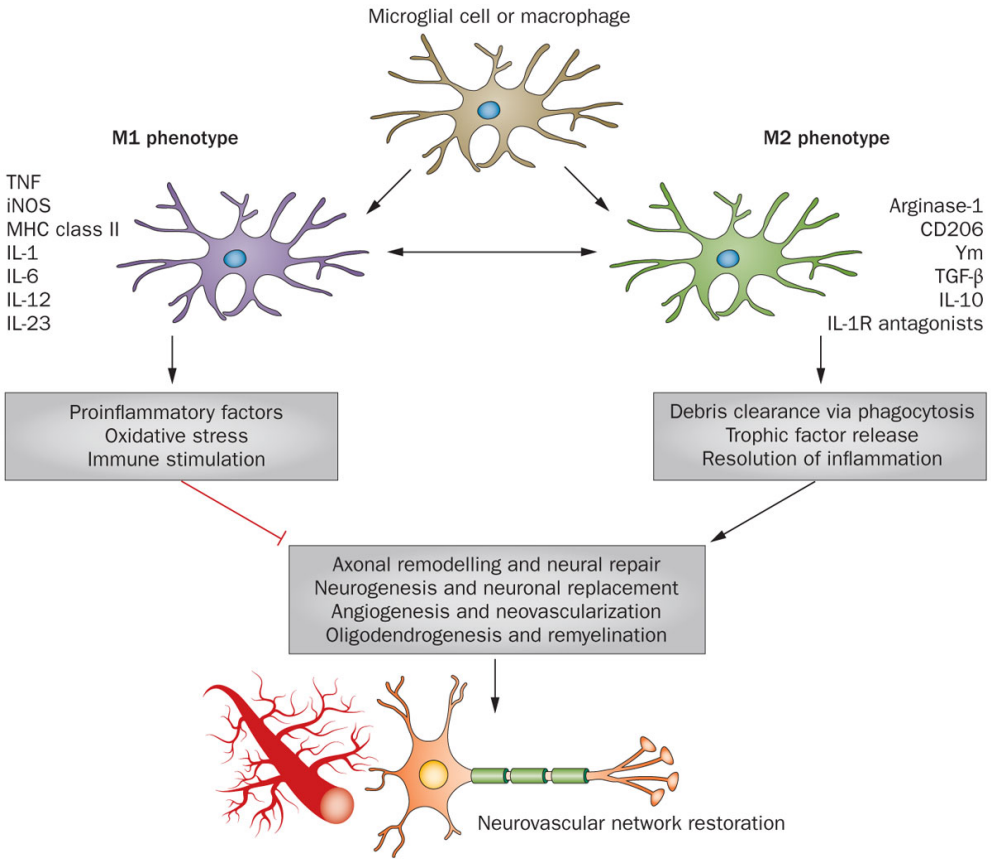


Figure 1.1 M1 vs. M2 microglial phenotypes

Hu, X. et al. Microglial and macrophage polarization—new prospects for brain repair.

Nat. Rev. Neurol. (2014) 11, 56–64

Oxidative and nitrosative stress signaling pathways in microglia

In general, M1 microglia are marked by activation state associated with the release of ROS, NO, glutamate, cytokines (such as TNFα), phospholipases, matrix metalloproteases, and other pro-inflammatory factors. Collectively, these products of inflammation can lead to progressive neuronal damage observed in many neuroinflammatory disorders (Takeuchi, Jin et al. 2006, Brown 2007, Chhor, Le Charpentier et al. 2013). By inference, suppressing or limiting microglial activation is

effective in preventing the development of neuroinflammation and subsequent neurodegeneration.

Both primary microglia and microglial cell lines can be activated with a variety of agents, such as proinflammatory cytokines (TNFα, IL-1β, IFNγ), lipopolysaccharides (LPS), and oligomeric beta amyloid (Aβ) (Hanisch 2002). Studies from our laboratory, as well as by others, well demonstrated microglia activation by LPS through the Toll-like receptor (TLR) and the NF-κB pathway. Our studies also show that IFNγ can independently activate microglia through the IFNγ receptor which is linked to stimulation of transcription factors such as interferon-gamma-activated sites (GAS), IFN regulatory factors (IRF), and the JAK-STAT pathway. In microglial cells, activation of NF-κB and GAS are necessary for induction of the iNOS gene. Since LPS and IFNγ could individually stimulate iNOS, our study provided evidence that these two pathways are regulated by a cross-talk mechanism involving p-ERK1/2 (Sheng, Zong et al. 2011). Inhibition of ERK1/2 by U0126, the inhibitor for MEK1/2, could completely abrogate induction of NADPH oxidase and iNOS by LPS and IFNγ (Sheng, Zong et al. 2011).

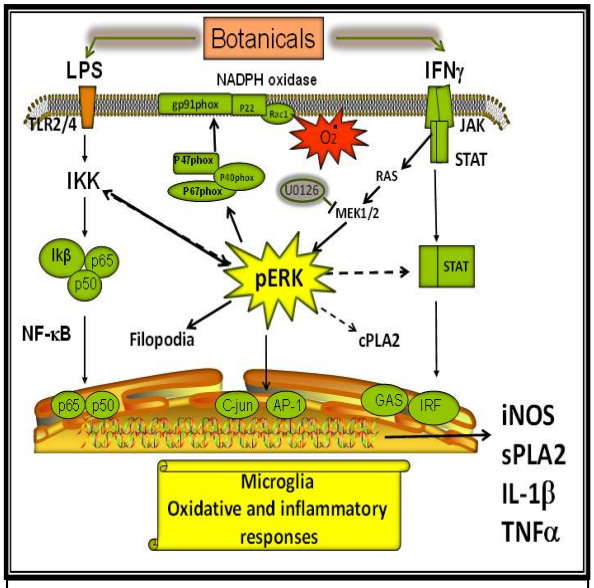


Fig. 1.2 LPS- and IFNγ-stimulated transcriptional pathways for activation of iNOS and ROS: role of p-ERK1/2 in mediating cross-talk between these two pathways.

NADPH oxidase is known to play a significant role in neurodegenerative diseases including alcohol-induced neurodegeneration (Qin and Crews 2012), Alzheimer's disease (Shimohama, Tanino et al. 2000), and Parkinson's disease (Wu, Teismann et al. 2003). This enzyme is comprised of both cytosolic and membrane bound sub-units and is present in different isoforms in a cell-specific manner.

While previous interest had focused on the role of this enzyme complex in neurons, NADPH oxidase is also a major source of ROS in the signaling transduction pathway in microglial cells (Vana, Li et al. 2011, Chuang, Chan et al. 2013). Superoxide anions generated by NADPH oxidase can react with NO to form peroxynitrite (ONOO-), which is a highly toxic radical with potent ability to damage cell membranes. Oxidation of polyunsaturated fatty acids (PUFAs) in membrane phospholipids can produce 4-hydroxy-2-nonenal (4-HNE), another reactive lipid peroxidation product which can form protein adducts (Shibata, Kato et al. 2011). Increase in 4-HNE is a good marker for assessing oxidative stress in injured brain tissue (Brennan, Suh et al. 2009).

Phospholipase A2

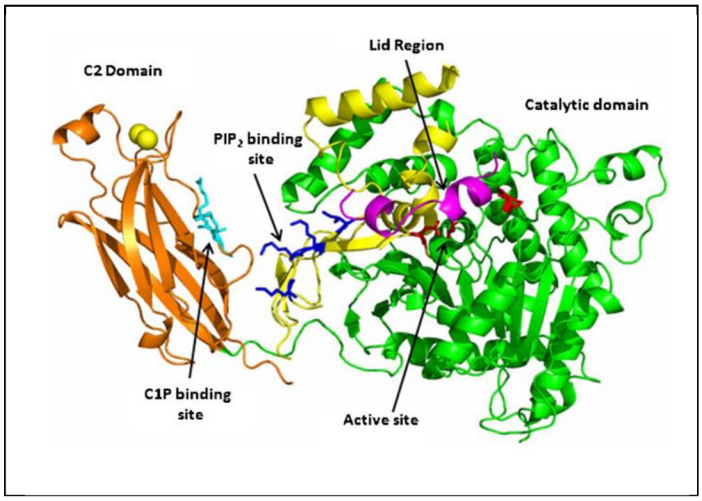
Phospholipids in the central nervous system (CNS) are enriched with polyunsaturated fatty acids (PUFAs), and phospholipases A2 (PLA2s) are the key enzymes for hydrolysis of the PUFAs in the sn-2 position of the glycerol moiety. More than 20 isoforms of PLA2s are present in mammalian cells, and are classified into three major categories, namely, secretory phospholipase A2 (sPLA2), calcium independent phospholipase A2 (iPLA2), and calcium-dependent (cytosolic) phospholipase A2

(cPLA2). In recent years, there is an increasing interest in uncovering the roles of different PLA2s in regulating cell functions under physiological and pathological conditions (Sun, Horrocks et al. 2007, Murakami, Taketomi et al. 2011, Sun, He et al. 2012). Arachidonic acid (AA) and docosahexaenoic acid (DHA) are two important PUFAs offering unique physiological functions to the brain. While activation of cPLA2 is linked to the release of AA, the type of PLA2 responsible for the release of DHA is less clear, although action of iPLA2 has been suggested (Cheon, Kim et al. 2012, Rapoport 2013). AA is substrate for cyclooxygenases and lipoxygenases, and serves as the precursor for the synthesis of eicosanoids and prostanoids that mediate a wide variety of inflammatory responses (Calder 2008). On the other hand, DHA is effective in mediating anti-inflammatory and neuroprotective responses (Cheon, Kim et al. 2012), and is the precursor for biosynthesis of neuroprotectin D (NPD1) and resolvins (Calder 2008, Niemoller and Bazan 2010, Palacios-Pelaez, Lukiw et al. 2010, Eady, Belayev et al. 2012, Mas, Croft et al. 2012, Orr, Palumbo et al. 2013).

cPLA2

The calcium-dependent group IV cytosolic PLA_2 ($cPLA_2\alpha$) is a 97 kDa protein constitutively expressed in nearly all brain cells. Investigation on $cPLA_2$ has gained special attention lately because not only is it required for calcium binding in the C2 domain, but can also undergo a number of post-translational modifications through phosphorylation and *S*-nitrosylation. In particular, several active serine/threonine residues are susceptible to phosphorylation by protein kinases that are involved in important signaling pathways, e.g., ERK1/2 and p38 MAPK at Ser505, Mnk1 at Ser727, and

CaMKII at Ser515 (Linkous and Yazlovitskaya 2010). In human epithelial cells, *S*-nitrosylation by nitric oxide (NO) of an active cysteine residue can enhance $cPLA_2$ activity by several fold (Xu, Han et al. 2008). $cPLA_2$ also contains cationic domains for binding zwitterionic lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) (Murakami, Taketomi et al. 2011), ceramide-1-phosphate (C1P), and lactosylceramide (Murakami, Taketomi et al. 2011, Nakamura, Moriyama et al. 2013, Ward, Bhardwaj et al. 2013). These properties suggest active modulation of this enzyme through interactions with lipids and intracellular signaling molecules.



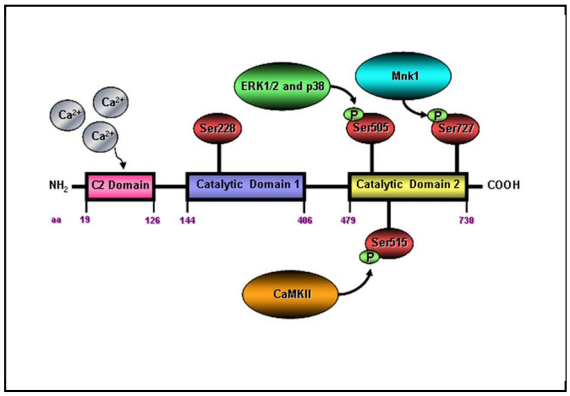


Fig. 1.3 Molecular structure of cPLA2 (Burke, JE & Dennis, EA, 2009)

In the CNS, activation of $cPLA_2$ has been implicated in the pathogenesis of a number of neurodegenerative disorders. Examples include experimental autoimmune encephalomyelitis (Kalyvas and David 2004), Alzheimer's disease (Kriem, Sponne et al. 2005, Sanchez-Mejia, Newman et al. 2008, Florent-Bechard, Desbene et al. 2009, Schaeffer, Forlenza et al. 2009, Sanchez-Mejia and Mucke 2010, Gentile, Reccia et al. 2012), Neimann-Pick disease (Nakamura, Yasufuku et al. 2012), tumorigenesis (Linkous, Yazlovitskaya et al. 2010), stroke (Bonventre, Huang et al. 1997, Saluja, O'Regan et al. 1999, Stephenson, Rash et al. 1999), alcoholism (Tajuddin, Przybycien-Szymanska et al.

2013, Moon, Tajuddin et al. 2014), as well as affective disorders (Rao, Kellom et al. 2012). However, few studies have elucidated the extracellular agonists and signaling pathways leading to cPLA$_2$ activation in these disease conditions.

cPLA2 in neurons

Neuronal stimulation by ionotropic glutamate receptor agonists, such as N-methyl-D-aspartic acid (NMDA), is known to elicit a massive and rapid influx of Ca^{2+} leading to activation of Ca^{2+}-dependent enzymes, inhibition of mitochondrial function and stimulation of neuronal apoptosis. Activation of the NMDA receptors is marked by a rapid production of reactive oxygen species (ROS) which is attributed to activation of NADPH oxidase (Shelat, Chalimoniuk et al. 2008, Brennan, Suh et al. 2009). Studies further linked NMDA-induced ROS production with activation of the MEK1/2-ERK1/2 pathway and phosphorylation of cPLA$_2$ (Shelat, Chalimoniuk et al. 2008). These findings demonstrated activation of cPLA$_2$ together with neuronal excitation and oxidative stress. In primary cultures of cortical and hippocampal neurons, treatment with a PLA$_2$ inhibitor, methylarachidonyl-fluorophosphonate (MAFP), resulted in altered neuronal morphology, and reduced neurite outgrowth and viability (Forlenza, Mendes et al. 2007). Stereotaxic injection of the PLA$_2$ inhibitor to the hippocampal CA1 area also caused a reduction of neuronal membrane fluidity which is thought to contribute to impairment in memory function (Forlenza, Schaeffer et al. 2007).

In the peripheral nervous system, cPLA$_2$ has been implicated in injury of primary sensory neurons and pain behavior (tactile allodynia) (Tsuda, Hasegawa et al. 2007). The

increase in $cPLA_2$ activity is attributed to stimulation of the ionotropic P2X receptors and subsequent activation of MAPK and CaMKII (Hasegawa, Kohro et al. 2009). In vascular smooth muscle cells, norepinephrine stimulated phosphorylation of $cPLA_2$ through activation of CaMKII, and phosphorylation of $cPLA_2$ by CaMKII (at S515) occurred prior to the phosphorylation by ERK1/2 (at S505). Complete phosphorylation by both kinases was required for AA release (Pavicevic, Leslie et al. 2008). There is further evidence that upon stimulation by norepinephrine, CaMKII promoted translocation of $cPLA_2$ from cytosol to the nuclear envelope (Fatima, Yaghini et al. 2003). In fact, $cPLA_2$ and CaMKII appeared to be present in close proximity in the dorsal root ganglion cells, and stimulation of the ganglion cells with ATP promoted the translocation of p-$cPLA_2$ to the plasma membrane (Hasegawa, Kohro et al. 2009). Since CaMKII is regarded an important protein kinase in modulation of synaptic plasticity and long-term potentiation (Liu and Murray 2012), these studies presented a possible link between CaMKII and $cPLA_2$ in modulating neuronal activity under disease conditions.

Organization of the pre- and post-synaptic structures is extremely complex. The presence of $cPLA_2$ in the synaptic area suggests a possible role for this enzyme in regulating synaptic activity. There is evidence that $cPLA_2$ modulates vesicle formation, for promoting neurite outgrowth and maintenance of growth cone activity. In the dorsal root ganglion, Semaphorin 3A (a class of secreted and membrane protein) can trigger a signaling pathway involving $cPLA_2$ activation, AA release and synthesis of 12(S)-hydroxyeicosatetraenoic acid (12(S) HETE), and in turn, mediates neuronal growth cone activity (Sanford, Yun et al. 2012). In agreement with the role of $cPLA_2$ in neuronal

membrane and synaptic activity, hippocampal neurons isolated from $cPLA_2$ knockout (KO) mice showed measurable differences in the nuclei and soma volume, and structure of the synaptic cleft as compared to neurons from wild-type animals (Qu, Gong et al. 2013). In the hippocampus and cerebellum, the PKC-ERK1/2-$cPLA_2$ pathway was implicated in long-term synaptic plasticity and regulation of AMPA receptor trafficking (Antunes and De Schutter 2012).

Albeit at lower levels as compared to stimulation by NMDA, oligomeric amyloid-beta peptide (Aβ) can also stimulate ROS production in neurons and subsequently, phosphorylate ERK1/2 and $cPLA_2$ (Shelat, Chalimoniuk et al. 2008). However, prolonged exposure of neurons to Aβ led to a decrease in NMDA response and an increase in mitochondrial dysfunction (He, Cui et al. 2011). Studies by Kriem's group demonstrated the ability for Aβ to activate $cPLA_2$ and subsequently mitochondrial dysfunction and neuronal apoptosis (Kriem, Sponne et al. 2005, Malaplate-Armand, Florent-Bechard et al. 2006). Besides stimulation of phospho-$cPLA_2$, Aβ exposure also led to activation of neutral sphingomyelinase and ceramide, and together, neuronal apoptosis (Sagy-Bross, Hadad et al. 2013). Mitigation of ROS production in neurons, such as that using gp91ds-tat, a specific inhibitor for the gp91phox subunit of NADPH oxidase, could protect neurons from the deleterious effects of Aβ (He, Cui et al. 2011). Studies with $cPLA_2$ deficient mice, together with different experimental models demonstrated the critical role for $cPLA_2$ in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and stroke (Chalimoniuk, Stolecka et al. 2009, Kishimoto, Li et al. 2010, Desbene, Malaplate-Armand et al. 2012, Last, Williams et al.

2012, Sundaram, Chan et al. 2012, Fang, Jiang et al. 2013). These studies further underscore studies on the role of $cPLA_2$ in neuronal metabolic signaling pathways linking to Ca^{2+} homeostasis, protein kinases activation, and oxidative-nitrosative stress.

cPLA2 in astrocytes

In astrocytes, ATP/UTP has the ability to stimulate G-protein-coupled $P2Y_2$ receptor and signaling pathways leading to activation of PKC-dependent and independent phosphorylation of ERK1/2 and $cPLA_2$ (Xu, Weng et al. 2002). Besides ATP, phorbol ester (PMA) can increase phospho-$cPLA_2$, AA release and production of prostaglandin E2 (PGE2) in astrocytes (Xu, Chalimoniuk et al. 2003). Studies have demonstrated a priming effect for pro-inflammatory cytokines (such as TNFα, IL-1β and IFNγ) to enhance the production of PGE2 following short term exposure to ATP and PMA (Xu, Chalimoniuk et al. 2003). Other agents such as lipopolysaccharides (LPS) can stimulate cPLA2 and PGE2 production through an ERK1/2-dependent pathway in astrocytes (Xiang, Chen et al. 2013). In fact, a number of other stimuli, including ammonia (Norenberg, Rama Rao et al. 2009), alcohol (Floreani, Rump et al. 2010), ceramide (Prasad, Nithipatikom et al. 2008), bradykinin (Hsieh, Wu et al. 2006, Hsieh, Wang et al. 2007), and diethylmaleate/iodoacetate (Liao, Ou et al. 2013) could stimulate $cPLA_2$ and engage in astrocytic inflammatory and oxidative pathways.

cPLA2 in microglia

In microglial cells, LPS stimulates $cPLA_2$ and PGE2 production through a signal transduction pathway involving sphingomyelinase and p38 MAPK (Akundi, Candelario-

Jalil et al. 2005). ATP also can induce $cPLA_2$ and PGE2 release from microglial cells and this action was attributed to the activation of P2X7 receptors (Anrather, Gallo et al. 2011). However, PGE2 production due to stimulation of P2X7 receptors appeared to involve COX1 and not COX2. In another study, infection of macrophages with *Candia albicans* resulted in rapid activation of $cPLA_2$, AA release, and production of eicosanoids, and the production of PGE2 was also mediated through COX1 (Suram, Silveira et al. 2013). Since COX1 is constitutively active in cells whereas COX2 is induced through the NF-κB pathway, rapid response of cPLA2 to agonists can cause inflammatory responses without involvement of the transcriptional processes. Chronic infusion of LPS to brains could cause microglial activation and inflammatory responses with increases in TNFα and iNOS. It is also observed that cPLA2 and LOX-5 were upregulated under this condition (Kellom, Basselin et al. 2012).

Studies from our laboratory have demonstrated the involvement of ERK1/2 in LPS-IFNγ-induced production of NO and ROS in microglial cells (Sheng, Zong et al. 2011, Chuang, Chan et al. 2013). In agreement with results from a study by Ribeiro et al. (2013), our study also indicated an increase in phospho-cPLA following LPS or IFNγ treatment (Ribeiro, Wen et al. 2013, Chuang, Simonyi et al. 2015). In rat primary microglial cells, an increase in the expression of total $cPLA_2$ occurred 6–8 hours after treatment with LPS (Vana, Li et al. 2011). In the BV-2 microglial cells, LPS-induced $cPLA_2$ activation involved ERK1/2 and JNK but not p38 MAPK (Ribeiro, Wen et al. 2013). Furthermore, $cPLA_2$ siRNA or its inhibitor, AACOCF3, attenuated LPS-induced NO and ROS production as well as iNOS and p67phox expression in microglial cells

(Vana, Li et al. 2011, Ribeiro, Wen et al. 2013). Taken together, these studies demonstrated the critical role of cPLA$_2$ in mediating inflammatory responses in microglial cells.

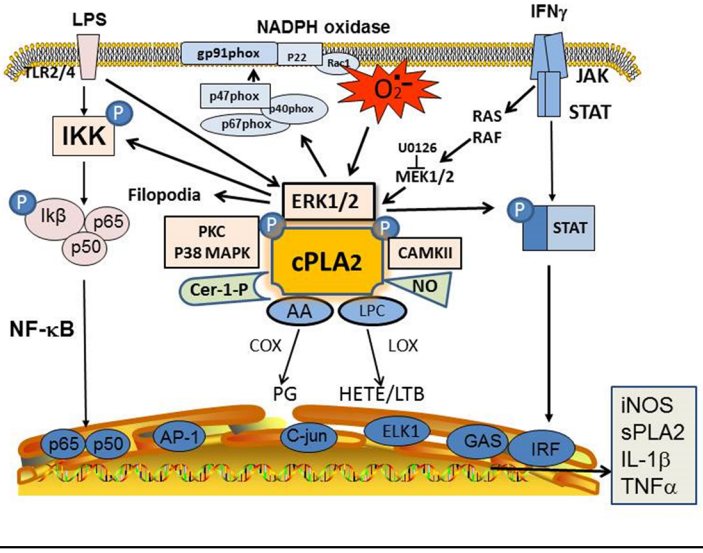


Fig. 1.4 cPLA2 in oxidative and inflammatory pathways in microglia cells. Sun et al., Role of cytosolic phospholipase A2 in oxidative and inflammatory signaling pathways in different cell types in the central nervous system. Mol. Neurobiology (2014) 50(1):6-14

Similar to neurons and astrocytes, aggregated Aβ can also confer toxic effects on microglial cells, as demonstrated by increased production of ROS and upregulation of phospho-cPLA$_2$ expression and cPLA$_2$ activity (Szaingurten-Solodkin, Hadad et al. 2009). Antisense cPLA$_2$ and pyrrophenone, a cPLA$_2$ specific inhibitor, were effective in abolishing ROS, iNOS and PGE2 production induced by Aβ.

Spinal microglial cells are activated during spinal cord injury and have been implicated in the pathogenesis of neuropathic pain (Ji and Suter 2007). Spinal microglial cells are susceptible to stimulation by LPS, which in turn induced the increase in COX-1

and COX-2 and production of PGE2 and NO through the p38 MAPK pathway (Matsui, Svensson et al. 2010). Interestingly, there is evidence for lysophosphatidic acid (LPA) receptors being involved in microglial stimulation upon spinal cord injury and neuropathic pain (Ma, Nagai et al. 2010). Besides activation of LPS1 and LPA3 receptors, this type of spinal cord injury and neuropathic pain also involve NMDA and neurokinin 1 receptors, as well as stimulation of $cPLA_2$ and $iPLA_2$ in microglial cells (Ma, Nagai et al. 2013). More studies are needed to investigate the underlying mechanisms linking spinal microglial cells and neurons in neuropathic pain.

cPLA2 as therapeutic target for CNS diseases

$cPLA_2$ has been regarded as a potentially viable target for intervention against neurological diseases involving neuroinflammation. A study by Sanchez-Mejia et al. compared transgenic hAPP mice with and without $cPLA_2$ knockout, and showed that mice deficient in cPLA2 had less cognitive deficits compared with those having intact cPLA2. In addition, primary neuronal cultures with cPLA2 knockout were more resistant to Aβ-toxicity, indicating a role of $cPLA_2$ in the pathogenesis of Alzheimer's disease (Sanchez-Mejia, Newman et al. 2008). By employing an experimental stroke model, Bonventre et al. also showed that $cPLA_2$ knockout mice suffered less ischemic damage and had smaller infarcts after transient middle cerebral artery occlusion followed by reperfusion (Bonventre, Huang et al. 1997).

AACOCF3, a non-selective $cPLA_2$ and $iPLA_2$ inhibitor, was found in the 1990's to be an effective pharmacological inhibitor of PLA_2. One of its strengths is its ability to

penetrate into cell membranes. In several studies, such as those using thrombin-stimulated platelets, Ca^{2+} ionophore-stimulated human monocytic cells, and interleukin 1-stimulated mesangial cells, AACOCF3 could effectively block AA production at a concentration of 5 to 20 μM (Gronich, Konieczkowski et al. 1994, Farooqui, Ong et al. 2006). Recent *in vivo* studies have utilized the administration of AACOCF3 to demonstrate the effects of cPLA2 inhibition against multiple neurological diseases. A study by Zhang et al. demonstrated protective effect from AACOCF3 treatment against focal ischemic damage in experimental stroke (Zhang, Barasch et al. 2012). Another study by Vana et al. showed prevention of secondary tissue damage in experimental autoimmune encephalitis, an *in vivo* model for multiple sclerosis (Vana, Li et al. 2011). Two additional studies revealed preservation of neuronal survival and retention of motor function in a mouse model of spinal cord injury (Huang, Bhavsar et al. 2009, Liu, Deng et al. 2014).

While most of the studies have focused on the action of $cPLA_2$ in neurons, especially in the event of neurodegeneration and neuronal apoptosis, less attention has been given to the potential role of $cPLA_2$ in the microglia cells. While microglial activation plays an important role in limiting neuronal damage and can phagocytose cellular debris and foreign pathogens, M1 activation of microglia cells can also induce neuronal damage and further propagate neuroinflammation. The results of this study shed light on a potential mechanism for the control and regulation of M1 microglia under pathological conditions. Interestingly, inhibition of $cPLA_2$ by AACOCF3 was able to abrogate the morphological changes elicited by LPS and IFNγ in WT primary microglial

cells. We believe that cPLA$_2$ inhibition not only prevents microglial activation, but more importantly, becomes a viable therapeutic strategy to impede neuronal cell death by limiting secondary neuronal damage. In this regard, inhibiting the PLA$_2$ cascade is an essential strategy for opposing microglia activation (Paris, Town et al. 2000) and discovering new and synthetic inhibitors for PLA$_2$ will be an important future endeavor for understanding and treatment of neurological disorders (Ong, Farooqui et al. 2015).

Medicinal plants for CNS diseases

Throughout human history, many natural products from plants have been shown to promote human health and manage disease symptoms, and some have been developed into modern-day drugs. One of the most notable cases was the recent awarding of Nobel Prize for Physiology or Medicine in 2015 to Youyou Tu, for her discovery of artemisinin (also known as qinghaosu, 青蒿素 in Chinese) and dihydroartemisinin, for the treatment of malaria.

Recent studies documented anti-oxidative and anti-inflammatory properties of polyphenols in fruits and vegetables and herbs, and their role in maintaining brain health during aging (Galli et al., 2002; Sun et al., 2008). During the past 5 years, studies by the MU Center for Botanical Interactive Studies (CBIS) further placed emphasis on dietary supplements including elderberries, garlic, soy and Sutherlandia. Making use of the BV-2 microglia cell line and rodent models of cerebral ischemia, studies in Dr. Sun and Dr. Gu's laboratories attempted to unravel effects of these botanicals on microglia oxidative

and inflammatory pathways as well as neuroprotection. The followings are brief descriptions of the botanicals involved in this dissertation.

Magnolia

The bark of Magnolia tree has been used as ingredients for traditional herbal medicine in Asian countries for hundreds of years (Lee, Lee et al. 2011). *Magnolia officinalis* (China) and *Magnolia obovata* (Japan) contain a rich source of biological active compounds, including alkaloids, coumarins, flavonoids, lignans, neolignans, and terpenoids (Ito, Iida et al. 1982, Tachikawa, Takahashi et al. 2000). Studies *in vitro* and *in vivo* have demonstrated strong potential for Magnolia extracts for treatment for a number of diseases, such as reducing allergic and asthmatic reactions, suppressing anxiety, and antagonizing angiogenic properties in cancers (Tsai, Huang et al. 1999, Ikarashi, Yuzurihara et al. 2001, Chang, Hsu et al. 2003, Liou, Shen et al. 2003, Lu, Chen et al. 2003, Zhou, Shin et al. 2008).

Fig. 1.5 *Magnolia officinalis*
(Copyright www.cnseed.org)

Of the active components identified in the bark of Magnolia, honokiol and magnolol are the two most studied polyphenolic compounds, and are isomers with similar properties. Recent *in vivo* studies on these compounds demonstrated beneficial effects against neurological disorders, namely anxiety, depression, Alzheimer's disease (AD),

Parkinson's disease (PD), and stroke. However, despite their apparent effectiveness in disease modification, the mechanism of action is still not well-understood (Watanabe, Watanabe et al. 1983, Maruyama, Kuribara et al. 1998, Liu, Hattori et al. 2005, Xu, Yi et al. 2008, Chang-Mu, Jen-Kun et al. 2010, Chen, Lin et al. 2011).

Studies in vitro have demonstrated anti-inflammatory effects of honokiol and magnolol in suppressing cytokine-induced NO production, expression of iNOS, and generation of prostaglandins and leukotrienes in macrophages, epithelial cells and renal mesangial cells (Oh, Kang et al. 2009, Kuo, Lai et al. 2010, Wu, Zhang et al. 2011). Neuronal excitation due to stimulation by the ionotropic glutamate receptor agonists is known to elicit a rapid influx of calcium, which triggers down-stream pathways leading to the production of ROS and mitochondrial dysfunction (Shelat, Chalimoniuk et al. 2008, Brennan, Suh et al. 2009). Understanding the underlying mechanism for Magnolia compounds to suppress neuronal excitotoxicity may help to explain their ameliorating actions in disease models.

Sutherlandia

Sutherlandia [*Sutherlandia frutescens* (L.) R. Brown or *Lessertia frutescens* (L.) Goldblatt & J.C. Manning], also known as cancer bush, is a native traditional medicinal plant widely used by population in the southern parts of African, including in South Africa, Namibia, Botswana, and Lesotho (Faleschini MT 2013). This botanical is used in

a variety of chronic ailments, including cancer, arthritis, digestive disorders, and diabetes, and more recently, in behavioral symptoms of HIV/AIDS and in depression and anxiety (Mills, Cooper et al. 2005, van Wyk and Albrecht 2008). Several studies have demonstrated antioxidant and anti-inflammatory properties for Sutherlandia extract in immune cells including microglia (Jiang, Chuang et al. 2014) and macrophages (Lei, Browning et al. 2015), as well as in many cancer cell lines (Fernandes, Cromarty et al. 2004, Ojewole 2004, Katerere and Eloff 2005, Kundu, Mossanda et al. 2005, Faleschini MT 2013, Jiang, Chuang et al. 2014).

Fig. 1.6 *Sutherlandia frutescens* (Copyright by Botanypictures.com)

While there is evidence to support Sutherlandia's benefit for mitigating stress (Prevoo, Smith et al. 2004) as well as drug-induced seizures (Ojewole 2008), little is known about its broader effects against neuro-inflammatory diseases. Studies done in our laboratory demonstrated dietary Sutherlandia mitigated neuronal damage from stroke *in vivo*, and suppressed the oxidative stress in activated microglia cells (Chuang, Cui et al. 2014).

Results from a randomized, double-blinded, placebo-controlled trial in healthy adults of consumption of Sutherlandia for 3 months showed it was well tolerated (Johnson, Syce et al. 2007). This was the first report to establish the safety for Sutherlandia consumption. Obviously, more studies are required to determine the effective dose and formally validate whether it does indeed lead to benefits in human health.

Elderberry

Consumption of elderberry, including the North American subspecies (*Sambucus nigra* L. subsp. *canadensis* [L.] Bolli), has increased in recent years, mainly for its claim to combat symptoms of common flu and other viral infections (Zakay-Rones, Varsano et al. 1995, Monograph 2005, Vlachojannis, Cameron et al. 2010). Elderberries are widely cultivated in Europe, Asia, North Africa, and North America (Monograph 2005). Elderberry fruit contains flavonoids and anthocyanins (Lee and Finn 2007) which are reported to have beneficial effects of human health, especially cardiovascular functions, anti-carcinogenic, anti-viral and anti-inflammatory effects (Prior and Wu 2006, Zafra-Stone, Yasmin et al. 2007). While elderberry pomace extracts exerted only small effects to mitigate LPS-stimulated NO and ROS in microglial cells, some of the metabolites, such as quercetin, are more active

Fig. 1.7 *Sambucus nigra*
(Copyright by hemlocklily.wordpress.com/)

(Simonyi, Chen et al. 2015, Sun, Chen et al. 2015). On the other hand, cyanidin, a flavonol with similar structure as quercetin, is less effective in inhibiting LPS-induced NO production. Rutin, the glycosylated form of quercetin, and cyanidin-3-O-glucoside are not active in suppressing oxidative and inflammatory responses in microglial cells (Sun, Chen et al. 2015). Aside from down-regulating the LPS-induced NF-κB pathway, our study further demonstrated the ability for quercetin to stimulate the antioxidant pathway involving Nrf2 and the antioxidant response element (ARE) which plays an important role in the transcriptional synthesis of a number of phase II detoxifying enzymes (Sun, Chen et al. 2015).

Cyanidin-3-glucoside is one of the most common anthocyanins in berries. Despite that it does not effectively suppress LPS-induced NO production in microglial cells, it is capable of exerting beneficial effects when administered in vivo, e.g., it ameliorated ethanol-induced neurotoxicity in developing brains, and protected against focal cerebral ischemia damage in mice (Ke, Liu et al. 2011, Min, Yu et al. 2011). There is further evidence suggesting the ability of these berries to prevent age-associated oxidative stress and improve neuronal and cognitive functions in animal models (Galli, Shukitt-Hale et al. 2002).

Summary and rationale for studies

Recent studies on neuroinflammation and neurodegenerative diseases place focus on the important role of microglial cells and the signaling pathways underlying the inflammatory responses. Our studies have placed focus on LPS-induced NF-κB and

IFNγ-induced JAK-STAT pathways and the role of ERK1/2 in mediating cross-talk between these pathways to induce ROS from NADPH oxidase and NO from iNOS. Furthermore, ERK1/2 also plays an important role in mediating phosphorylation of cPLA2, an enzyme important in modifying membrane phospholipids and production of arachidonic acid for biosynthesis of eicosanoids. Along with studies to unveil signaling pathways and mechanism for inflammatory responses in microglial cells, studies also investigate effects of botanical polyphenols and extracts.

Experiments and specific aims outlined in this dissertation:

(1) To examine effects of botanical polyphenols (honokiol and magnolol) on microglial oxidative and inflammatory response to stimulation by LPS and IFNγ. This study has been published in the Journal of Neuroinflammation by Chuang et al., titled Magnolia polyphenols attenuate oxidative and inflammatory responses in neurons and microglial cells (Chuang, Chan et al. 2013).

(2) With limited information available for understanding the role of cPLA2 in mediating oxidative/inflammatory responses in microglial cells, a study was launched to examine cPLA2 in both immortalized and primary microglial cells and its response to LPS and IFNγ. This study has recently been published in Journal of Neuroinflammation by Chuang et al., titled Cytosolic phospholipase A2 plays a crucial role in ROS/NO signaling during microglial activation through the lipoxygenase pathway (Chuang, Simonyi et al. 2015).

(3) Investigating effects of botanical polyphenols to suppress cPLA2 in microglial cells and subsequent mitigation of microglial-derived neuro-toxic effects using the differentiated SH-SY5Y neuroblastoma cells as a model. This manuscript has been reviewed and pending on minor revision, and will be published in a special issue of NeuroMolecular Medicine.

(4) An animal model of cerebral ischemia was used to investigate whether the proposed anti-inflammatory and anti-oxidative properties of these botanicals can translate to mitigate neuronal cells death and ischemic damage from acute neuroinflammation due to ischemia/reperfusion after stroke. This study was published in ASN Neuro by Chuang et al., titled Dietary Sutherlandia and elderberry mitigate cerebral ischemia-induced neuronal damage and attenuate p47phox and phospho-ERK1/2 expression in microglial cells (Chuang, Cui et al. 2014).

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CHAPTER 2

MAGNOLIA POLYPHENOLS ATTENUATE OXIDATIVE/NITROSATIVE STRESS IN NEURONS AND MICROGLIAL CELLS

ABSTRACT

Background

The bark of Magnolia has been used in Oriental medicine for treatment of a variety of remedies including some neurological disorders. Magnolol (Mag) and Honokiol (Hon) are isomers of polyphenolic compounds from the bark of *Magnolia officinalis*, and have been identified as major active components exhibiting anti-oxidative, anti-inflammatory and neuro-protective effects. In this study, we investigate ability for these isomers to suppress oxidative/nitrosative stress in neurons stimulated by the ionotropic glutamate receptor agonist N-methyl-D-aspartate (NMDA) and in microglial cells activated by interferon-γ (IFNγ) and lipopolysaccharide (LPS). Attempts were also made to elucidate the mechanism and signaling pathways involved in cytokine-induced production of reactive oxygen species (ROS) in microglial cells.

Methods

Dihydroethidium (DHE) was used to assay superoxide production in neurons and CM-H2DCF-DA was used to test for ROS production in murine (BV-2) and rat (HAPI) immortalized microglial cells. NADPH oxidase inhibitors (e.g., DPI, AEBSF and

apocynin) and immunocytochemistry targeting p47phox and gp91phox were used to assess involvement of NADPH oxidase. Western blot was used to assess iNOS and ERK1/2 expression, and the Griess reaction protocol was employed to determine nitric oxide (NO) concentration.

Results

Exposure of Hon and Mag (1-10 μM) to neurons for 24 h did not alter neuronal viability, but both compounds (10 μM) inhibited NMDA-stimulated superoxide production, a pathway known to involve NADPH oxidase. In microglial cells, Hon and Mag inhibited IFNγ±LPS-induced iNOS expression, NO and ROS production. Studies with inhibitors and immunocytochemical assay further demonstrated the important role of IFNγ activating the NADPH oxidase through the p-ERK-dependent pathway. Hon and to a lesser extent, Mag, inhibited IFNγ-induced p-ERK1/2 and its down-stream pathway for ROS and NO production.

Conclusion

This study underscores the important role of NADPH oxidase in mediating oxidative/nitrosative stress in neurons and microglial cells and unveiled the role for IFNγ to stimulate the MAPK/ERK1/2 signaling pathway for activation of NADPH oxidase in microglial cells. Hon and Mag offer anti-oxidative/nitrosative effects, at least in part, through suppressing IFNγ-induced p-ERK1/2 and its down-stream pathway.

INTRODUCTION

The bark of Magnolia tree has been used in a number of traditional herbal medicinal preparations in Oriental countries for thousands of years (Lee et al., 2011). *Magnolia officinalis* (China) and *Magnolia obovata* (Japan) contain a rich source of biological active compounds, including alkaloids, coumarins, flavonoids, lignans, neolignans, and terpenoids (Ito et al., 1982; Tachikawa et al., 2000). According to *in vitro* and *in vivo* studies, and recent clinical trials, there is strong evidence for these constituents to play an important role in the treatment for a plethora of ailments by reducing allergic and asthmatic reactions, and suppressing anxiety and angiogenic responses (Tsai et al., 1999; Ikarashi et al., 2001; Chang et al., 2003; Liou et al., 2003b; Lu et al., 2003; Zhou et al., 2008).

Magnolol (Mag) and Honokiol (Hon) (Fig. 2.1) are polyphenolic compounds from *Magnolia officinalis* belonging to the neolignan family. Recent evidence for these compounds to exert beneficial effects in neurological disorders, such as anxiety, depression, stroke, Alzheimer's disease (AD), and Parkinson's disease (PD) has attracted great attention to further investigate their molecular mechanism and specific targets (Watanabe et al., 1983; Maruyama et al., 1998; Liu et al., 2005; Xu et al., 2008; Chang-Mu et al., 2010; Chen et al., 2011). Neuronal excitation due to stimulation by the ionotropic glutamate receptor agonists is known to elicit a rapid influx of calcium which triggers down-stream pathways leading to the production of ROS and mitochondrial dysfunction (Shelat et al., 2008; Brennan et al., 2009). Understanding the underlying

mechanism for Magnolia compounds to suppress neuronal excitotoxicity may help to explain their ameliorating actions in disease models.

Studies with cell models have demonstrated anti-inflammatory effects of Hon and Mag in mitigating cytokine-induced NO production, expression of iNOS, and generation of prostaglandins and leukotrienes (Oh et al., 2009; Kuo et al., 2010; Wu et al., 2011). This type of inflammatory response is important in microglial cells because their activation has been the basis of a number of neurodegenerative diseases. Although cytokines and LPS have been shown to activate microglial cells and induce ROS and NO production, mechanistic details within the signaling pathways leading to this type of oxidative/nitrosative stress have not been clearly elucidated.

In this study, we aim to test the ability for Hon and Mag to suppress oxidative/nitrosative stress in neurons and microglial cells. Studies with neurons were based on the excitotoxic model demonstrating the involvement of NAPDH oxidase in NMDA-stimulated ROS production (Shelat et al., 2008). Studies with microglial cells demonstrated that NADPH oxidase was also involved in mediating cytokine and LPS-induced ROS production. In addition, our studies further unveiled the important role of the IFNγ - ERK1/2 signaling pathway for ROS production and ability for Hon and Mag to suppress this pathway in microglial cells.

MATERIALS AND METHODS

Materials

Honokiol (lot number M8P0236) and magnolol (lot number M8F3374) (≧98% pure based on HPLC) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). These compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solutions. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, 0.05% (w/v) trypsin/EDTA, and phosphate-buffered saline (PBS) were obtained from GIBCO (Gaithersburg, MD). Interferon-γ (IFNγ) was purchased from R & D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) (rough strains) from Escherichia coli F583 (Rd mutant) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, MO). AlamarBlue™ kit was from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA). Antibodies used for Western blot included: goat anti-rabbit IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase and iNOS polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-β-actin peroxidase (Sigma-Aldrich, St. Louis, MO); ERK1/2, phospho-ERK1/2, (Cell Signaling, Beverly, MA). Antibodies used for immunocytochemical staining include rabbit anti-p47phox antibodies (Calbiochem, Billerica, MA), mouse anti-gp91phox (Thermo Fisher, Waltham, MA), goat-anti-rabbit Alexa fluor 488 (Jackson Immunoresearch, West Grove, PA), and goat-anti-mouse Alexa fluor 549 (Jackson Immunoresearch, West Grove, PA). For ROS detection, CM-H2DCFDA (DCF) was obtained from Invitrogen, Inc. (Carlsbad, CA), and dihydroethidium (DHE) from Sigma-Aldrich (St. Louis, MO). Inhibitors used in this study include: MEK inhibitor U0126 (Cell Signaling, Beverly, MA), 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF,

Calbiochem, San Diego, CA), diphenyleneiodonium (DPI) and apocynin (Sigma-Aldrich, St. Louis, MO).

Cell culture

Preparations of primary cortical neuron cells involved pregnant E17 Sprague-Dawley rats (Harlan, IN, USA). All animal care and experimental protocols were carried out in accordance with NIH guidelines and with permission from the University of Missouri Animal Care and Use Committee (protocol #6728). Primary cortical neurons were prepared from the cerebral cortices of E17 Sprague-Dawley rat embryos as described (He et al., 2011). Briefly, cerebral cortices were dissected and meninges removed. The tissues were suspended in 3 ml 0.05% (w/v) trypsin/EDTA and incubated for 30 min at 37°C. The cell suspension was triturated through fine-burned-tip glass pipette until tissues were homogenized. The filtrate was centrifuged at 1000 g for 1 min and re-suspended in 10% FBS in DMEM containing 100 units/ml penicillin and streptomycin (100 μg/ml). Finally, cells were plated in 24-well plates for MTT analysis and 35mm dishes for ROS detection. The plates were pre-coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) the day before plating and incubated overnight. Four hours after plating, culture medium was completely changed to B27 supplemented neurobasal medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and glutamine. Culture was maintained by changing 1/2 volume of B27 medium in each well every 4 days. Experiments were conducted 8 days after plating for adequate culture maturation.

The immortalized mouse BV-2 and rat HAPI microglial cells were cultured as described previously (Shen et al., 2005). Briefly, cells were cultured in 75 cm^2 flasks with DMEM (high glucose) supplemented with 10% FBS containing 100 units/ml penicillin and 100 µg/ml streptomycin, and maintained in 5% CO_2 incubator at 37°C. For subculture, cells were removed from the culture flask with a scraper, re-suspended in the culture medium and sub-cultured in 12/24/96-well (1.0×10^6) plates for experiments.

Assessing cell viability

Two assay protocols were used to assess neuron viability after exposure to Hon and Mag. Since mitochondrial dysfunction is an initial step in apoptotic pathways that lead to neuronal cell death, the MTT assay was used for determination of mitochondrial dysfunction. In this assay, neurons were cultured in 24-well plates and treated with Hon or Mag (1 – 10 µM) at 37ºC for 24 h. After treatment, culture medium was removed and 1 ml of MTT reagent (0.5 mg/ml), dissolved in serum free DMEM, was added to each well. The plates were incubated for 3 h at 37°C, and the formazan particles in each well were dissolved in 500 µl of DMSO. After shaking the plates at room temperature for 5 min, absorbance was read at 540 nm using a Synergy4 Plate Reader (BioTek Instruments, Inc, Fisher Scientific, St. Louis, MO, U.S.A.).

AlamarBlue™ is a cell permeable non-fluorescent dye, which can be converted into red fluorescence upon reductive reactions within live cells. This assay was used to determine the extent of neuronal viability after exposure to Hon and Mag (1 – 10 µM). In this assay, neurons were cultured in 24-well plates and then treated with Hon or Mag

(1 – 10 μM) at 37°C for 24 h. After treatments, 100 μl of AlamarBlue™ was added to each well and neurons were further incubated at 37°C for 3 h. Absorbance was read at 570 nm using the Synergy4 Plate Reader (BioTek) with measurement at 600 nm as a reference.

Measurement of superoxide in neurons and ROS production in microglial cells

Dihydroethidium (DHE) is oxidized by superoxide anions to produce fluorescent ethidium, which is intercalated into DNA, and can be quantified by summing the fluorescence within the cell (Chapman et al. 2005). This protocol has been successfully used to measure superoxide production in neurons (Shelat et al., 2008; He et al., 2011). In brief, neurons were cultured on 35 mm dishes pre-coated with poly-L-lysine. Neurons were then treated with Hon or Mag (10 μM) for 30 min, and followed by exposure to NMDA (100 μM) for 30 min in phenol red free Neurobasal medium with 0.5 mg/ml BSA. At 30 min prior to image acquisition, cells were loaded with DHE (10 μM) and incubated at 37˚C. Fluorescence images were acquired using a Nikon TE-2000 U inverted microscope with a 20× NA 0.95 objective and a cooled CCD camera controlled with a computer running the MetaView imaging software (Universal Imaging, West Chester, PA). The fluorescence excitation source was controlled with a Uni-Blitz mechanical shutter. For image acquisition, a short exposure time (200 msec) and low intensity excitation light were applied to minimize photo-bleaching. Digital images were analyzed using the MetaView software with automatic background subtraction. Threshold fluorescence was obtained for each image prior to the quantification. For each field, the total fluorescence was measured and expressed as average fluorescence

normalized by the total number of cells. For each treatment group, at least three random images from the same dish were captured and analyzed, and each treatment was repeated three times independently for statistical analysis.

For measurement of ROS production in microglial cells, we adopted the protocol using CM-H2DCF-DA, a compound which becomes fluorescent upon interacting with ROS including H_2O_2 (Forkink et al., 2010). In this study, microglial cells were seeded in a 96-well plate, and after they became 90% confluent, they were serum-starved for 4 h. Cells were treated with cytokines and/or LPS for different times and CM-H2DCF-DA (10 μM) was added 1 h before measurement. In some experiments, cells were pretreated with U0126 or Hon/Mag for 1 h prior to stimulating with IFNγ for 11 h, and followed by adding the CM-H2DCF-DA (10 μM) and further incubated for 1 h. The fluorescent intensity of DCF was measured using the Synergy4 microplate reader with excitation wavelength of 490 nm and emission wavelength of 520 nm.

Measurement of NO

Cells were serum-starved in phenol red free DMEM for 3 h, followed by pretreatment of compounds of interest for 1 h. Cells were then treated with IFNγ and LPS or IFNγ alone and incubated at 37˚C for 16 h. NO released from cells was converted to nitrite in the culture medium, which was determined using the Griess reagent protocol. In brief, aliquots (50 μl) of culture medium were transferred to a 96-well plate and incubated with 50 μl of Reagent A (1% sulfanilamide in 5% phosphoric acid) per well for 10 minutes at room temperature covered in dark. This was followed by incubation with 50 μl

of reagent B (0.1%, w/v, N-1-napthylethylenediamine dihydrochloride, Sigma-Aldrich) per well for 10 minutes at room temperature covered in dark. Serial dilutions of sodium nitrite (0-100 μM) were used to generate the nitrite standard reference curve. Following the incubation period, absorbance at 543 nm was measured using the Synergy4 microplate reader.

Western blot analysis

Cells were harvested in a lysis radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. The extract was centrifuged at 10,000 × g for 15 min at 4°C to remove cell debris. Protein concentration was determined with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). For each sample, 5 μg of protein was loaded and resolved in 8% SDS-PAGE and run at 100 V. After electrophoresis, proteins were transferred to 0.45 μm nitrocellulose membranes at 300 mA for 3 h. Membranes were blocked in Tris-buffered saline (TBS), pH 7.4, with 0.1% Tween 20 (TBS-T) containing 5% non-fat milk for 1 h at room temperature. For different experiments, the blots were incubated with ERK1/2 (1:2000), phospho-ERK1/2 (1:2000), iNOS polyclonal (1:1000) antibodies overnight at 4°C. After repeated washing with TBS-T, blots were incubated with goat anti-rabbit IgG- horseradish peroxidase (1:4000) or goat anti-mouse IgG- horseradish peroxidase (1:2000) for 1 h at room temperature. The blots were then washed three times with TBS-T. Immunolabeling was detected by chemiluminescence ECL/WestPico/femto. For loading control, blots were incubated with monoclonal anti-β-actin peroxidase (1:30,000). For quantification, blots were scanned and the intensity of

protein bands was measured as optical density using the QuantityOne program (BioRad, Hercules, CA).

Immunostaining of p47phox and gp91phox in microglial cells

BV-2 microglia cells were serum-starved for 4 h, followed by stimulation with IFNγ (10 ng/mL) for 12 h. The cells were fixed in 4% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 in PBS for 30 min. Cells were incubated with 10% normal goat serum in 0.005% Triton X-100 in PBS for 60 min. Cells were then incubated overnight in 0.5% normal goat serum in 0.005% Triton X-100 in PBS containing primary antibodies: rabbit anti-p47phox antibodies (1:500; Calbiochem) and mouse anti-gp91phox antibodies (1:500; Thermo Fisher). The next day, cells were incubated in 0.005% Triton X-100 in PBS containing secondary antibodies, goat-anti-rabbit Alexa fluor 488 (Jackson Immunoresearch) and goat-anti-mouse Alexa fluor 549 (Jackson Immunoresearch) for 60 min, followed by nuclear counterstaining in PBS containing 1 μg/ml of using 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Pierce) for 10 min. The coverslips were then mounted on fluoromount (Sigma-Aldrich) and sealed with nail polish. Fluorescence photomicrographs were captured using a Leica DMI 6000B fully automated epifluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL) as serial z-stack images and process for deconvolution with AF6000 applications.

Statistical analysis

Data are presented as mean ± SEM. Results were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests or two-way ANOVA with Bonferroni posttests (V4.00; GraphPad Prism Software Inc., San Diego, CA). Statistical significance was considered for $p < 0.05$.

RESULTS

Hon and Mag suppressed NMDA-induced superoxide production in neurons

Since our earlier study had demonstrated the involvement of NADPH oxidase in the production of superoxide upon stimulating neurons with the ionotropic glutamate receptor agonist, NMDA (Shelat et al., 2008), this protocol was used to examine ability for Hon and Mag to suppress neuronal oxidative event induced by NMDA. The MTT assay was used to mark mitochondrial dysfunction and the AlamarBlue™ assay for assessing neuron cell death. As shown in Fig. 2.2A, treatment of neurons with Hon and Mag (1- 10 μM) for 24 h neither altered mitochondrial function nor causeed neuron cell death.

Subsequently, we tested the effects of Hon and Mag on superoxide production in neurons stimulated with NMDA (100 μM for 30 min). Treatment of neurons with NMDA elicited a large increase in superoxide production (Fig 2.2B, C). Hon and Mag (10 μM) alone did not elicit superoxide production in neurons; however, both compounds significantly inhibited NMDA-induced superoxide production (Fig 2.2B, C).

Hon and Mag inhibited IFNγ- and LPS-induced iNOS expression and NO production in BV-2 microglial cells

Similar to our previous study (Sheng et al., 2011), treatment of BV-2 microglial cells with IFNγ + LPS caused the induction of iNOS expression and a correlative increase in NO production (Fig. 2.3). Hon and to a lesser extent, Mag, inhibited LPS+IFNγ-induced iNOS expression and NO production in BV-2 cells in a dose-dependent manner (Fig. 2.3). Significant inhibition of NO ($p<0.05$) by Hon was observed at 1 μM, whereas significant inhibition for Mag was observed at 6 μM. Western blot analysis also indicated a dose-dependent inhibition of iNOS protein expression by Hon and Mag (Fig. 2.3B, C).

Similar to results observed by our earlier study (Sheng et al., 2011), addition of IFNγ to BV-2 microglial cells greatly stimulated LPS-induced iNOS expression and NO production. Under this condition, Hon and to a lesser extent, Mag, could dose dependently inhibit the induction of iNOS and NO induced by IFNγ (Fig. 2.4A - C). In fact, inhibition of IFNγ-induced NO production was similar to that using LPS + IFNγ (Fig. 2.3A).

In order to confirm the effects of Hon and Mag on IFNγ-induced NO production in other types of microglial cells, the rat HAPI microglial cells were tested. As shown in Fig. 2.5A, similar results were obtained for Hon to inhibit IFNγ-induced NO in HAPI cells. On the other hand, Mag appeared to provide greater inhibition in HAPI cells as compared to BV-2 cells (Fig. 2.5B).

ROS production in BV-2 microglial cells

Although previous studies have demonstrated the ability for LPS + IFNγ to induce ROS production in microglial cells, little is known about the source and the mechanism of action of the cytokines. In this study, a time course experiment was carried out to examine ROS production upon treating BV-2 microglial cells with LPS and/or IFNγ. Data in Fig. 2.6A showed no detectable ROS during the initial 4 h after exposing cells to LPS and/or IFNγ. LPS alone showed a slow increase in ROS after 4 h and peaked at 16 h, whereas IFNγ induced about 50% more ROS as compared with LPS. When cells were treated with IFNγ together with LPS, there was a left shift for ROS production although the peak level was similar to that induced by IFNγ alone (Fig. 2.6A).

Based on the time course conditions, we used the 12 h time point to examine whether Hon and Mag may alter IFNγ-induced ROS production. Results indicated ability for Hon and a lesser effect for Mag to inhibit IFNγ-induced ROS production in a dose-dependent manner. However, neither compound alone could exert obvious effects on the basal ROS production (Fig. 2.6B and C).

NAPDH oxidase is involved in IFNγ-induced ROS production

We further investigated whether NADPH oxidase may be the target enzyme for the ROS induced by IFNγ in microglial cells by testing with inhibitors commonly used for NADPH oxidase. Results show that DPI, a non-specific oxidase inhibitor, and AEBSF, a serine protease inhibitor (Diatchuk et al., 1997), could inhibit IFNγ-induced

ROS in microglial cells in a dose-dependent manner (Fig. 2.7A and B).

Using these inhibitors, we further tested whether IFNγ-induced ROS is critical for down-stream signaling pathway leading to NO production. As shown in Fig. 2.7C and D, DPI and AEBSF similarly inhibited IFNγ-induced NO production. Apocynin, a botanical compound known to inhibit translocation of cytosolic subunits p47phox and p67phox to the membrane complex (Stolk et al., 1994), was shown to inhibit IFNγ-induced NO production albeit at high concentrations starting at 40 μM (Fig. 2.7E).

Activation of NADPH oxidase is known to act through a complex mechanism involving activation of cytosolic subunits (p47phox, p67phox, p40phox, rac) to dock with the membrane subunits (gp91phox, p22phox). In the past, several types of kinases have been shown to phosphorylate the cytosolic subunits (p47phox, p67phox, p40phox) (Bokoch et al., 2009). In this study, double immunocytochemical staining was used to demonstrate that IFNγ could induce the translocation of p47phox from the cytosol to dock with the gp91phox in the membrane (Fig. 2.8), further supporting the involvement of the NADPH oxidase in the ROS production.

IFNγ-induced phosphorylation of MAPK/ERK1/2

Although cytokines and LPS were shown to induce phosphorylation of ERK1/2, it is not clear whether this response is a reflection of IFNγ (Sheng et al., 2011). Therefore, a time course assay was carried out to examine ability for IFNγ to induce p-ERK1/2. IFNγ induced a steady increase in p-ERK1/2 expression which was noticeable around 1 h and reaching a plateau after 4 h (Fig. 2.9A-C). Hon, and to a lesser extent, Mag,

inhibited IFNγ-induced ERK1/2 phosphorylation in a dose-dependent manner (Fig. 2.10A-C). Similarly, Hon also inhibited IFNγ-induced pERK1/2 in HAPI cells (Fig. 2.11A-C)

IFNγ-induced increase in p-ERK1/2 is upstream of ROS and NO production

In order to test whether IFNγ-induced p-ERK1/2 is up-stream of ROS and NO production, the MEK1/2 inhibitor, U0126, was used to block phosphorylation of ERK1/2 by MEK1/2. As shown in Fig. 2.12A, U0126 effectively inhibited IFNγ-induced p-ERK1/2 in BV-2 cells. U0126 also inhibited IFNγ-induced ROS production in a dose-dependent manner whereas U0126 did not influence basal ROS production in BV-2 cells (Fig. 2.12B). Under similar conditions, U0126 was shown to inhibit IFNγ-induced NO production in BV-2 and HAPI cells (Fig 2.12C).

DISCUSSION

In this study, we used rat primary cortical neurons and immortalized microglial cells (murine BV-2 and rat HAPI) to demonstrate the ability of Hon and Mag to suppress oxidative/nitrosative stress induced by NMDA in neurons and IFNγ±LPS in microglial cells. Exposure of neurons to Hon and Mag (up to 10 μM) for 24 h did not alter neuronal viability, but both compounds (10 μM) dramatically inhibited NMDA-induced superoxide production. Results from earlier studies have demonstrated involvement of NADPH oxidase in neuronal excitation (Shelat et al., 2008; Brennan et al., 2009), and Mag and Hon were shown to offer protection against neuronal toxicity induced by

hydrogen peroxide and ionotropic glutamate receptor agonists (Lin et al., 2006). These and other results are in line with the suggestion of possible therapeutic application of these compounds to treat neurological disorders (Lee et al., 2011).

The induction of iNOS expression and NO production by pro-inflammatory cytokines and LPS is known to involve the transcriptional pathways associated with NF-κB and the canonical JAK/STAT pathway induced by IFNγ (Marcus et al., 2003; Shen et al., 2005). Interestingly, in BV-2 and HAPI microglial cells, LPS and IFNγ can individually stimulate iNOS/NO production, suggesting presence of cross-talk mechanisms between these two pathways (Otero et al., 2007; Mir et al., 2009; Sheng et al., 2011). There is evidence for signaling molecules such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) to mediate the cross-talk pathways (Shen et al., 2005; Chao et al., 2010). In studies with microglial cells, an increase in MAPK-ERK1/2 activity has been shown to occur during induction of iNOS by LPS and IFNγ (Chan and Riches, 2001; Blanchette et al., 2003; Herrera-Molina et al., 2012). Our results here also demonstrated the ability for IFNγ alone to stimulate phosphorylation of ERK1/2 in microglial cells (Fig. 2.9A, B). However, unlike studies with neurons showing a rapid induction of p-ERK1/2 in minutes in response to NMDA stimulation (Shelat et al., 2008), phosphorylation of ERK1/2 by IFNγ in BV-2 microglial cells turned out to be a much slower and gradual process, first observable after 1 h and reaching a plateau around 4 h. In human macrophages, stimulation of p-ERK1/2 by IFNγ required an even more delayed time of 6 h (Smyth et al., 2011). Apparently, the time required for ERK1/2 phosphorylation varies depending on the cell types and agonists used for activation (Saud

et al., 2005).

Although pro-inflammatory cytokines have been shown to stimulate ROS production in microglial cells, the mechanism(s) for ROS production has not been clearly elucidated (Zhao et al., 2011). In our study with microglial cells, ROS production induced by LPS and IFNγ was not observed before 4 h (Fig. 2.6A). Since IFNγ-induced ROS production was inhibited by the MEK inhibitor (U0126, Fig. 2.12B), it is reasonable to conclude that p-ERK1/2 plays an important role in the induction process. Our results are in line with earlier studies demonstrating the involvement of ERK1/2 for IFNγ to stimulate superoxide in a mixed glial cell culture (Herrera-Molina et al., 2012). Our study further provided evidence for involvement of NADPH oxidase in IFNγ-induced ROS production in microglial cells. Inhibitors such as DPI and AEBSF not only inhibited ROS but also NO production, suggesting that ROS production precedes induction of NO. In order to further demonstrate the involvement of NADPH oxidase in IFNγ-induced ROS production, double immunocytochemical-staining showed translocation of p47phox from cytoplasm to membrane and co-localized with the gp91phox after treating cells with IFNγ (Fig. 2.8). In a study with macrophages, IFNγ was shown to activate NADPH oxidase through an increase in intracellular trafficking and expression of gp91phox (Casbon et al., 2012).

Many extracellular signals are coupled with cell surface receptors linking to activation of the MAPK pathway. In this study, we demonstrated the role for IFNγ to stimulate ROS from NADPH oxidase through the MEK-ERK1/2 pathway. Indeed,

activation of ERK1/2 has been shown to elicit multiple downstream events, including phosphorylation of cytosolic phospholipase A2 (cPLA2) and arachidonic acid release in neurons (Shelat et al., 2008), production of filopodia in BV-2 microglial cells (Sheng et al., 2011), transcytosis of macromolecules across the epithelial monolayers (Smyth et al., 2011), and enhancing phosphorylation of STAT1 (Li et al., 2010).

Taken together, these results demonstrated a novel role for IFNγ to stimulate the MAPK-ERK1/2 pathway, and in turn, to activate ROS production through the NADPH oxidase. Since IFNγ induced p-ERK is upstream of ROS and iNOS/NO production, agents that inhibit p-ERK1/2 can shut down both ROS production and iNOS induction. Understanding this mechanism helps to explain the earlier observation that LPS + IFNγ induced ROS production in microglial cells preceded the induction of iNOS (Pawate et al., 2004).

Many natural polyphenols, including luteolin, gastrodin, and ginsenoside, exhibit anti-inflammatory properties and inhibit LPS/IFNγ-induced NO production in microglial cells either through ERK1/2 or other types of MAPK (Chang et al., 2003; Liou et al., 2003a; Jung et al., 2009; Jung et al., 2010; Dai et al., 2011; Kao et al., 2011). Other studies have demonstrated the ability of Hon and Mag to inhibit cytokine-induced NO production, expression of iNOS, as well as generation of prostaglandins and leukotrienes (Kang et al., 2008; Oh et al., 2009; Kuo et al., 2010; Wu et al., 2011). Our studies unveiled the role for IFNγ-ERK1/2 pathway for ROS production through NADPH oxidase, and ability for Hon and Mag to suppress this oxidative/nitrosative mechanism in

microglial cells. Understanding this mechanism can help to explain the ability for these botanicals to suppress hyperactivity in brain and retard microglial cell activation and ameliorate neuroinflammatory responses associated with neurodegenerative diseases.

SUMMARY/CONCLUSION

In summary, our studies with neurons and microglial cells provided strong evidence for anti-oxidative/nitrosative effects of Hon and Mag and underscore the important role of NADPH oxidase in the superoxide/ROS production in these cells. Results with microglial cells further unveiled the important role of IFNγ in stimulating signaling pathways involving activation of ERK1/2, ROS and NO. These results also provide a useful and novel platform for testing anti-oxidative/nitrosative effects of other botanicals.

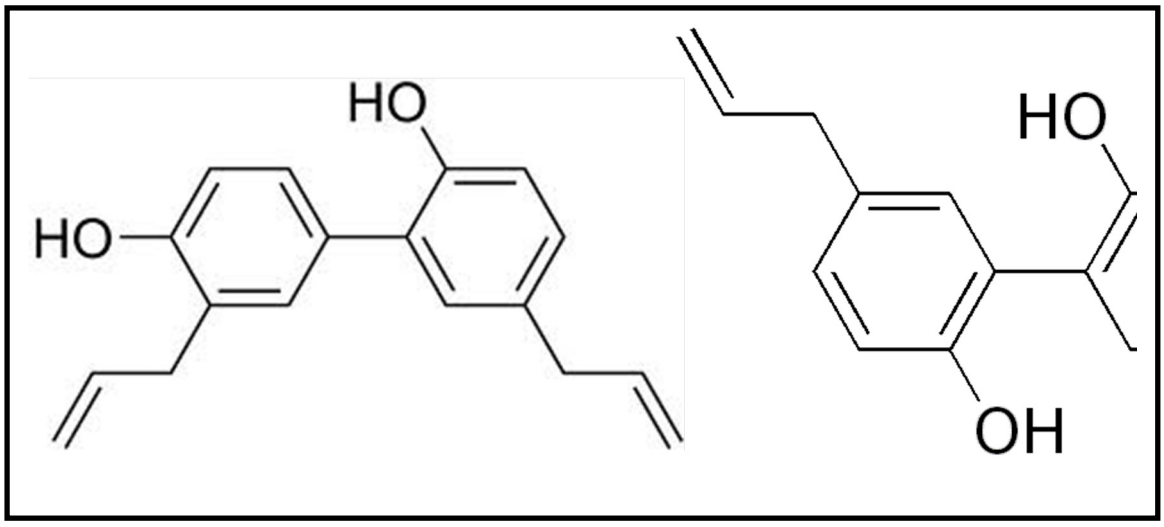
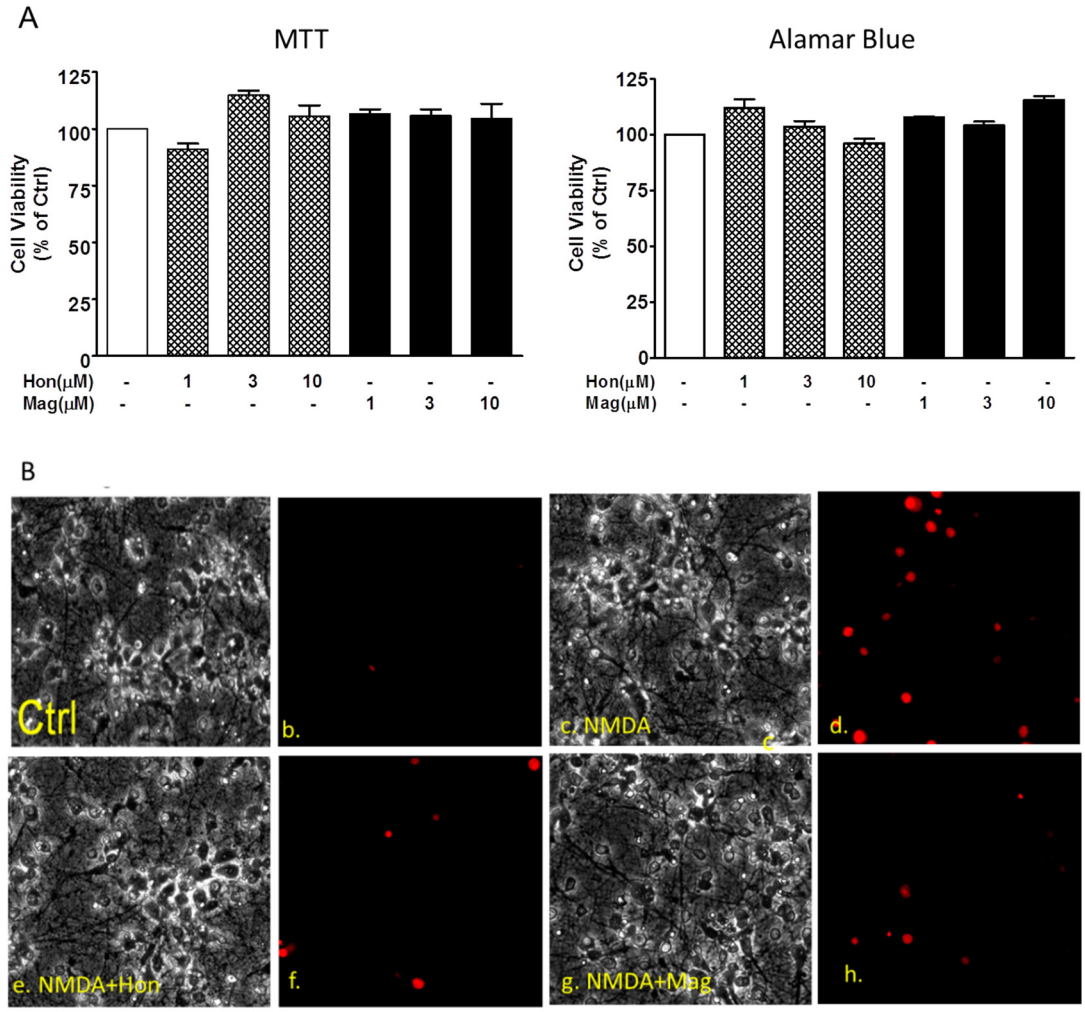


Figure 2.1
Structure of honokiol and magnolol


A
MTT
Alamar Blue
Cell Viability (% of Ctrl)
125
100
75
50
25
0
Hon(μM) - 1 3 10 - - -
Mag(μM) - - - - 1 3 10
B
Ctrl
b.
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d.
e. NMDA+Hon
f.
g. NMDA+Mag
h.
C
Average fluorescence (fold of Ctrl)
20
16
12
8
4
0
**
##
##
Ctrl
NMDA
Hon
Hon + NMDA
Mag
Mag + NMDA

Figure 2.2

Hon and Mag do not alter cell viability and inhibit NMDA-induced ROS production in primary rat cortical neurons. (A) Exposure of Hon and Mag (1-10 μM) to primary cortical neurons for 24 h did not alter neuronal viability as assayed by MTT and AlamarBlue. (B) Representative bright field and DHE fluorescence photomicrographs depicting ROS production in primary neurons after cells were treated with Hon or Mag (10 μM) for 30 min prior to stimulation with NMDA (100 μM) for 30 min. For ROS production, neurons were loaded with dihydroethidium (DHE, 10 μM) 30 min prior to image acquisition under different treatment conditions. Procedure for fluorescence determination is described in text. (C) Bar graph of average fluorescence depicting significant inhibition of NMDA-induced ROS production by Hon and Mag. Data are expressed as the mean $\pm$ SEM from 3 individual experiments and analyzed by two-way ANOVA with Bonferroni posttests. ** denote significant difference between control and NMDA ($p<0.001$), ## indicate significant decrease in ROS production by Hon and Mag as compared to NMDA ($p<0.01$).

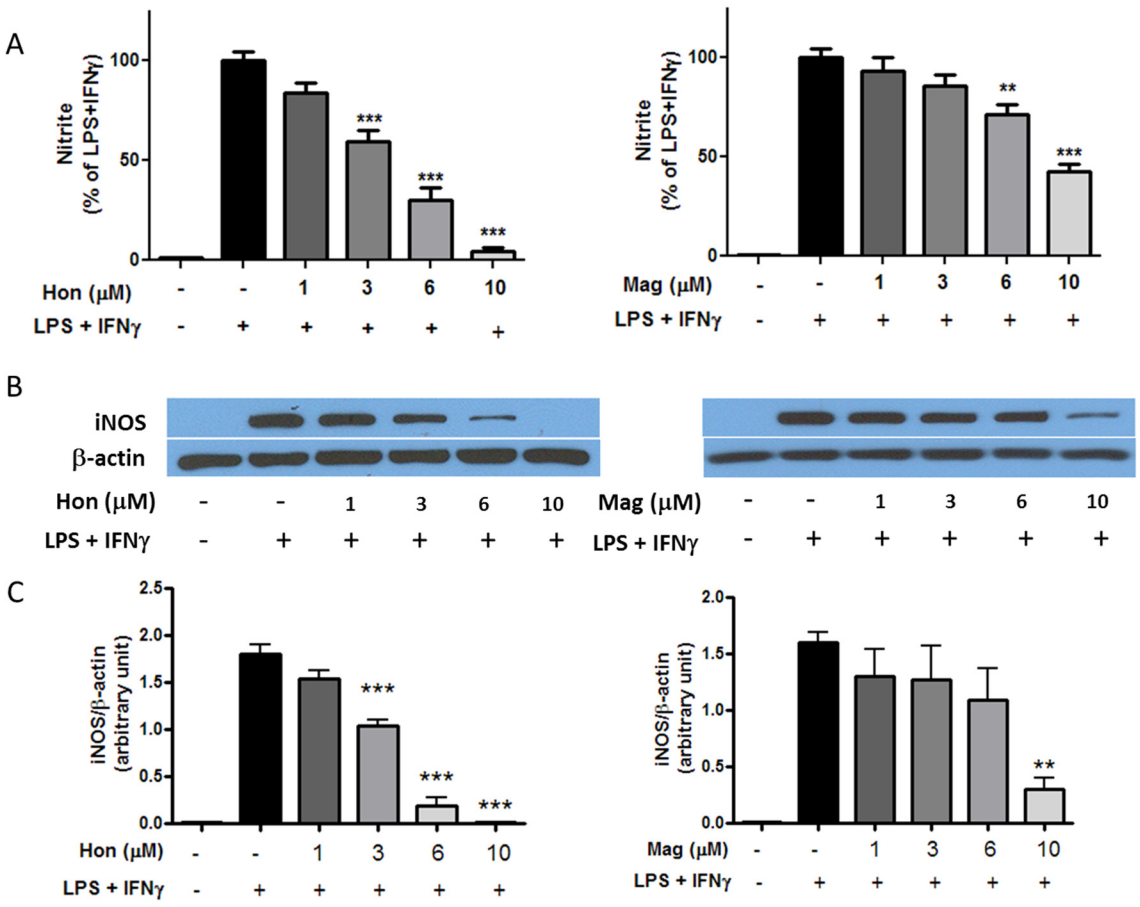

Figure 2.3

Hon and Mag inhibit LPS + IFNγ-induced NO production and iNOS expression in BV-2 microglial cells. Cells were treated with Hon or Mag (1-10 μM) for 1 h followed by stimulation with LPS (100 ng/ml) + IFNγ (10 ng/ml) for 16 h. (A) Culture media were collected for determination of NO using the Griess reaction protocol as described in text. (B) Representative Western blot of iNOS protein and β-actin. (C) Bar graphs representing iNOS/β-actin ratios. Results were expressed as the mean ± SEM (n = 3) and significant difference from the respective LPS + IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, ** $p<0.01$; ***$p<0.001$.

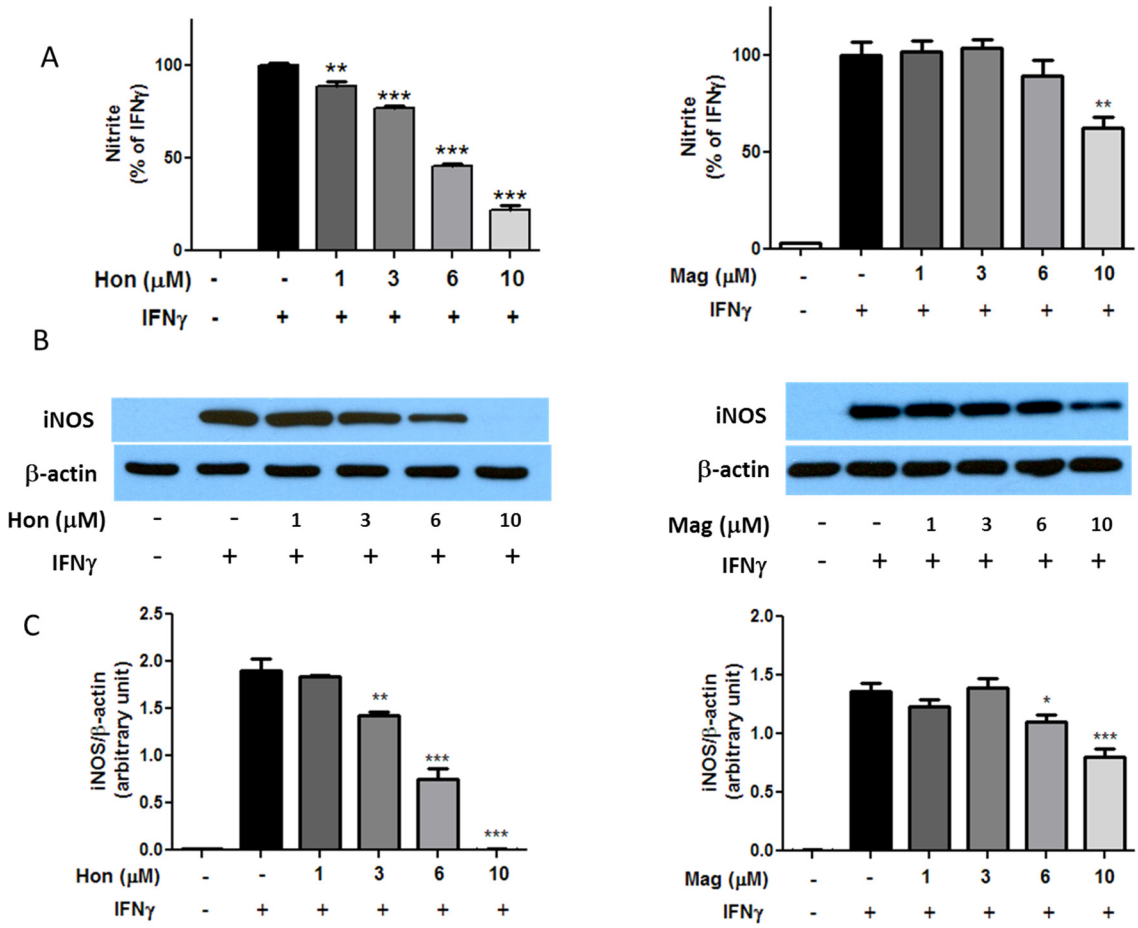

Figure 2.4

Hon and Mag inhibit IFNγ-induced NO production and iNOS expression in BV-2 microglial cells. Cells were treated with Hon or Mag (1-10 μM) for 1 h followed by stimulation with IFNγ (10 ng/ml) for 16 h. (A) Culture media were collected for determination of NO using the Griess reaction protocol as described in text. (B) Representative Western blots of iNOS protein and β-actin. (C) Bar graphs representing iNOS/β-actin ratios. Results were expressed as the mean ± SEM (n = 3) and significant difference from the respective IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05, ** p<0.01, ***p<0.001.

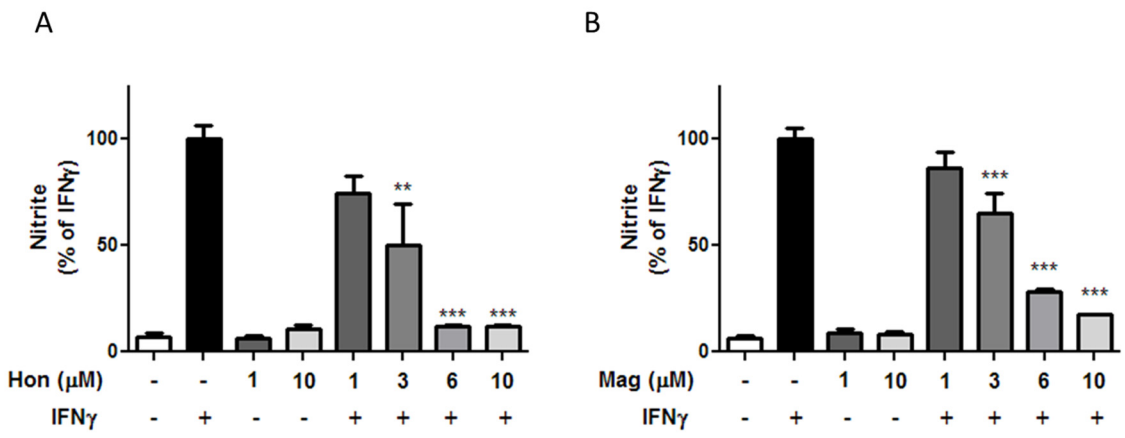

Figure 2.5

Hon and Mag inhibit IFNγ-induced NO production in HAPI microglial cells. Cells were treated with (A) Hon or (B) Mag (1-10 μM) for 1 h followed by stimulation with IFNγ (10 ng/ml) for 16 h. Culture media were collected for determination of NO using the Griess reaction protocol as described in text. Results were expressed as the mean ± SEM (n = 3) and significant difference from the respective IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05, ** p<0.01, ***p<0.001.

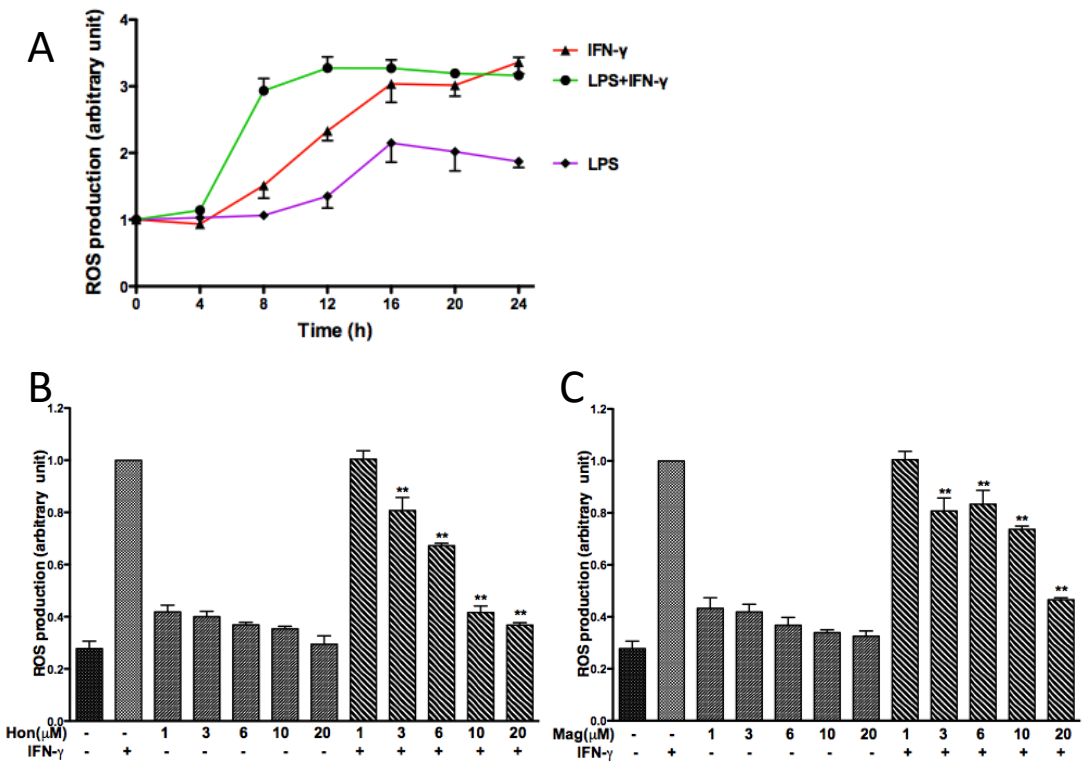

Figure 2.6

Hon and Mag inhibit cytokine-induced ROS production in BV-2 microglial cells. (A) Time course for ROS production induced by LPS and/or IFNγ. Cells were treated with LPS (100 ng/ml) and/or IFNγ (10 ng/ml) for the time indicated. ROS production was measured using CM-H2DCFDA as described in text. Similarly, (B) Hon and (C) Mag (1-10 μM) were given 1 h prior to exposure to IFNγ (10 ng/ml) for 12 h. Results are expressed as the mean ± SEM (n = 3) and significant difference from the respective IFNγ-stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05; ** p<0.01

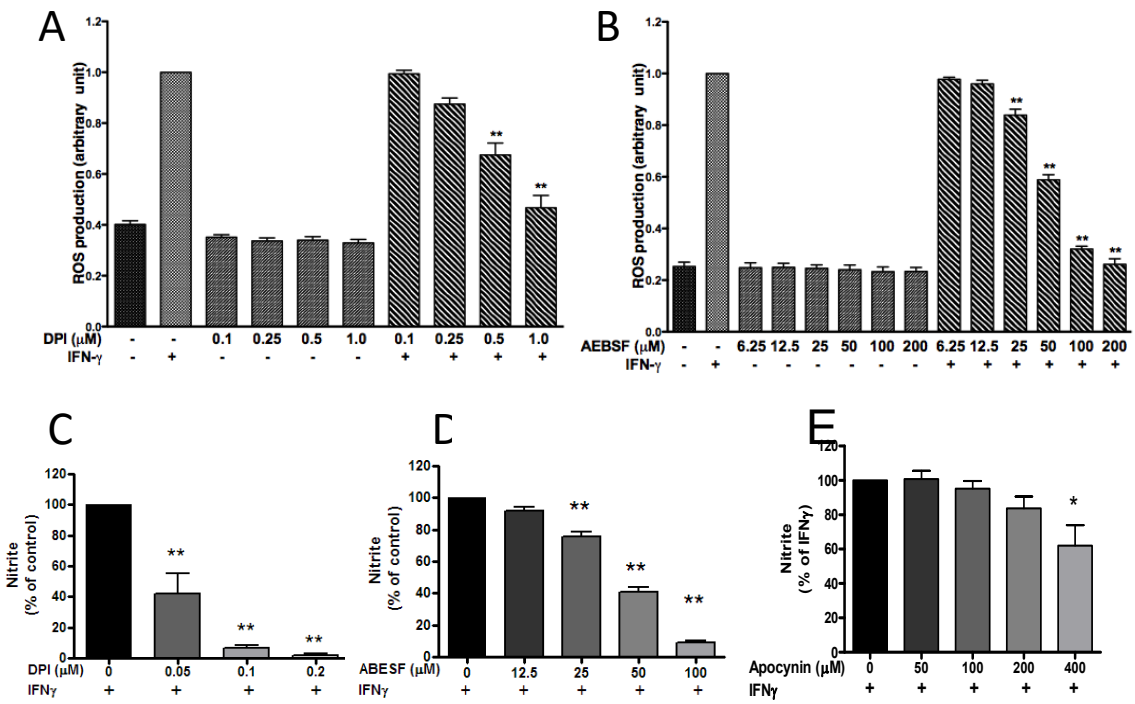

Figure 2.7

Activation of NADPH oxidase and ROS production is upstream of NO production in BV-2 microglial cells. Cells were pretreated with (A) DPI (0.1 – 1.0 μM) or (B) AEBSF (6.25 - 200 μM) for 1 h prior to exposure to IFNγ (10 ng/ml) for 12 h, followed by measure of ROS with CM-H2DCFDA as described in text. Cells were pretreated with (C) DPI (0.05 – 0.2 μM), (D) AEBSF (12.5 - 100 μM), or (E) Apocynin (50 - 400 μM) for 1 h prior to exposure to IFNγ (10 ng/ml) for 16 h, followed by measure of nitrite with Griess reaction protocol as described in text. Results are expressed as the mean ± SEM (n = 3), and significant difference from the respective IFNγ-stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05; **p<0.01

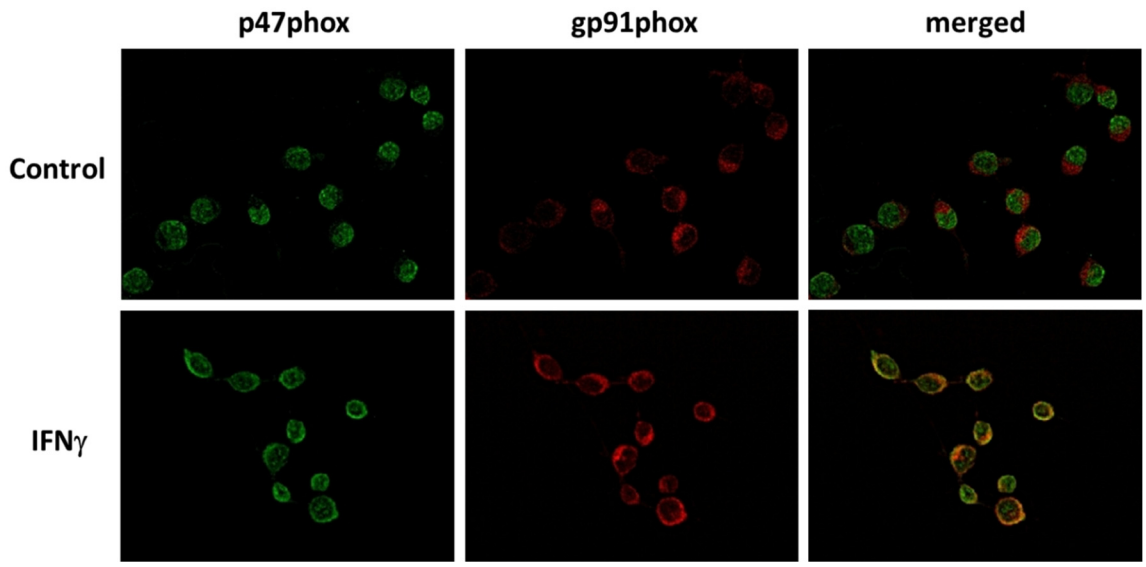

Figure 2.8

Involvement of NADPH oxidase in IFNγ-induced NO production in BV-2 microglial cells. Cells were exposed to IFNγ (10 ng/ml) for 12 h. Immunocytochemistry was performed with antibodies against p47phox (green) and gp91phox (red). Images were taken under fluorescence microscope as serial z-stack images, followed by deconvolution processing.

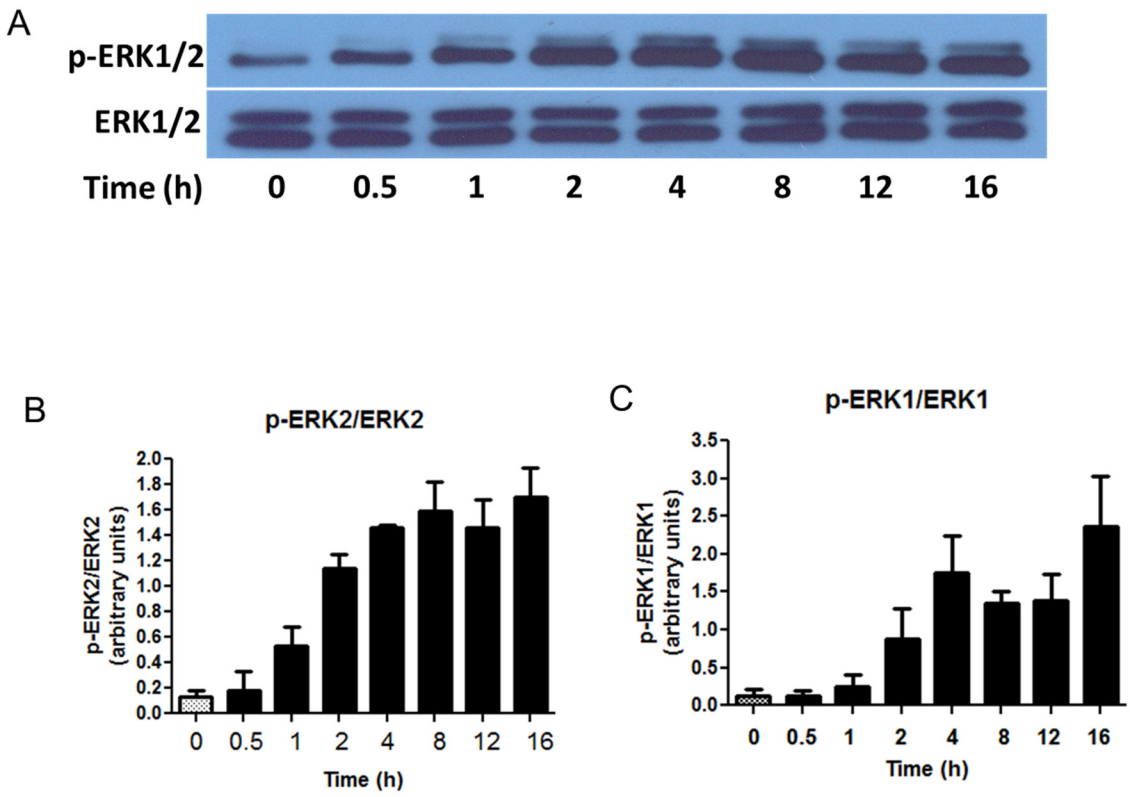

Figure 2.9

Time course of IFNγ-induced activation of p-ERK1/2 in BV-2 microglial cells. (A) Western blot analysis of a typical time course for IFNγ (10 ng/ml) to induce ERK1/2 phosphorylation in BV-2 microglia cells. Cell lysates were extracted at the time indicated. (B & C) Results of protein band intensities are expressed as arbitrary units of phospho-ERK1/2 against total ERK1/2 for (B) ERK1 and (C) ERK2. Results are expressed as the mean $\pm$ SEM (n = 3).

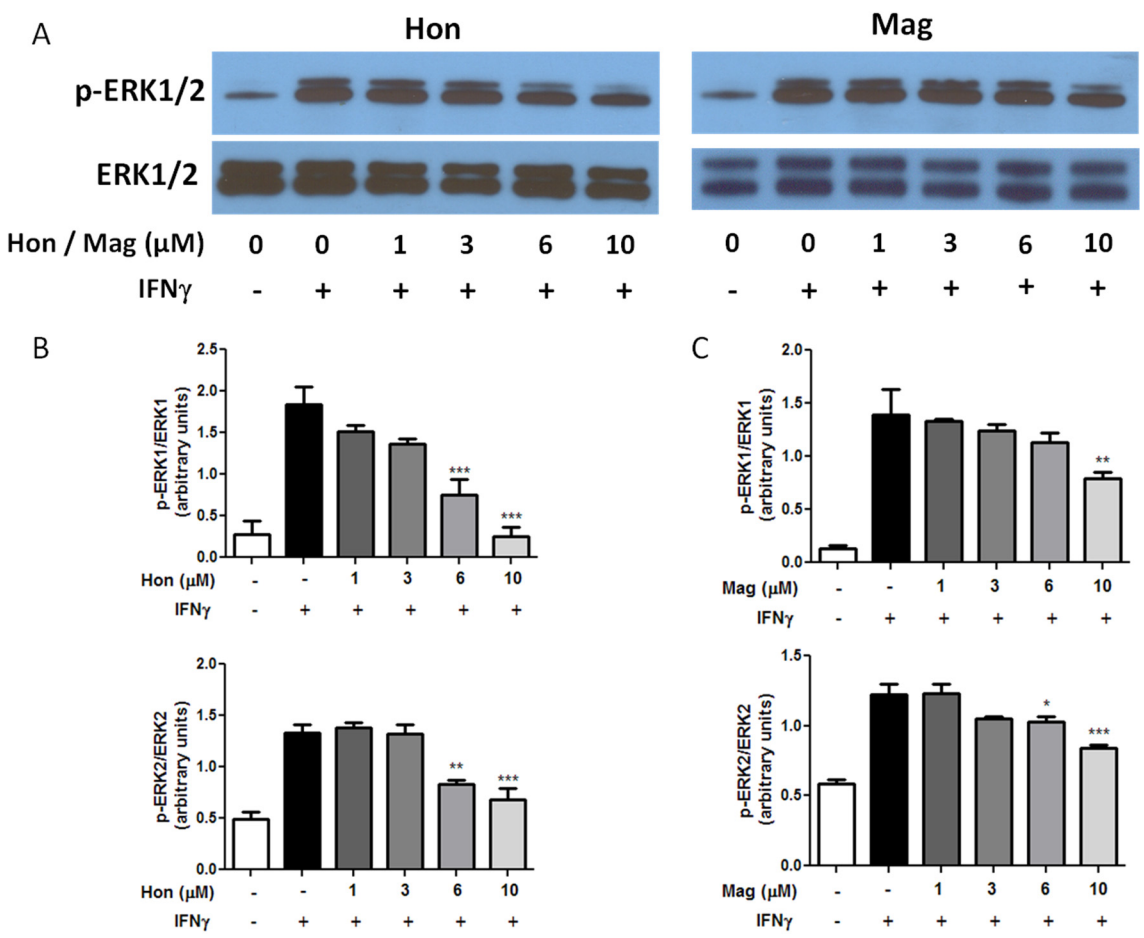

Figure 2.10

Hon and Mag inhibit IFNγ-induced activation of p-ERK1/2 in BV-2 microglial cells. (A) Western blot analysis showing a representative experiment of Hon or Mag pretreatment on IFNγ-induce p-ERK1/2 phosphorylation in BV-2 microglia cells. Cells were treated with either Hon or Mag (1- 10 μM) for 1 h followed by stimulation with IFNγ (10 ng/ml) for 4 h. (B & C) Results of protein band intensities are expressed as arbitrary units of phospho-ERK1/2 against total ERK1/2. Results are expressed as the mean ± SEM (n = 3) and significant difference from the respective IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05; ** p<0.01; *** p<0.001.

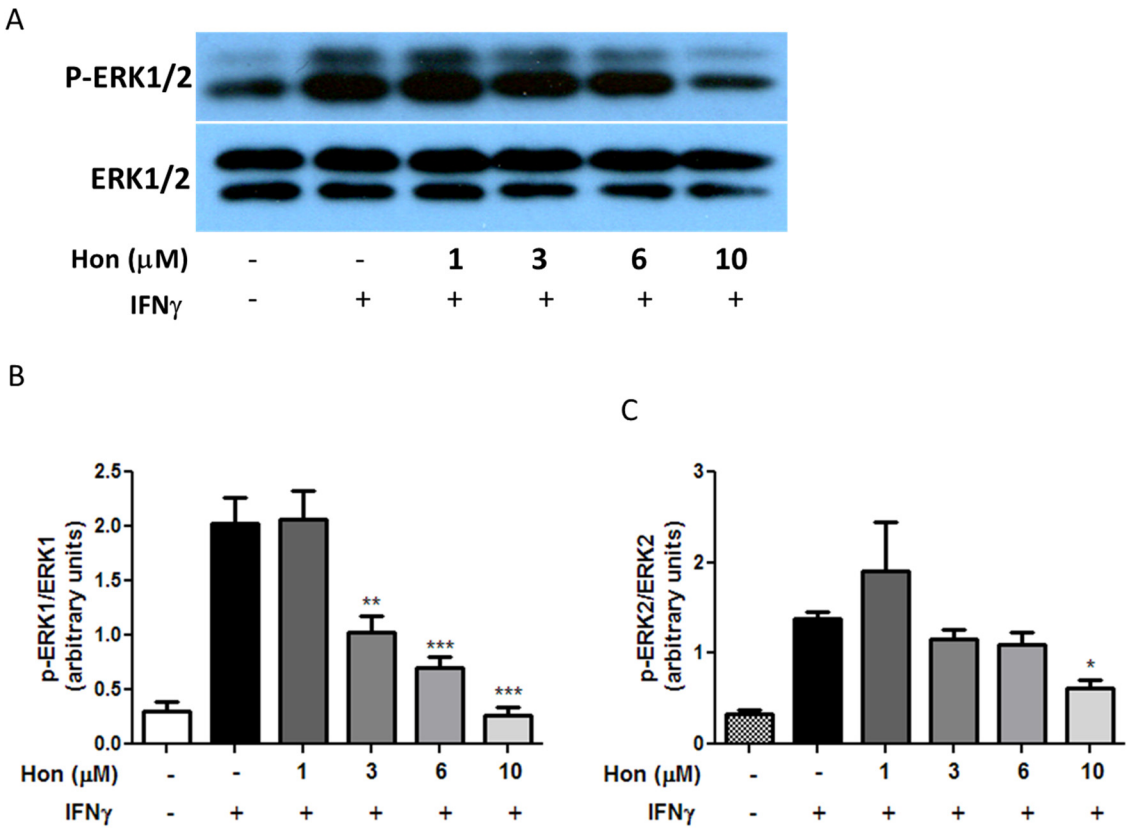

Figure 2.11

Hon inhibits IFNγ-induced activation of p-ERK1/2 in HAPI microglial cells. (A) Western blot analysis showing a representative experiment of Hon pretreatment on IFNγ-induceERK1/2 phosphorylation in HAPI microglial cells. Cells were treated with Hon (1- 10 μM) for 1 h followed by stimulation with IFNγ (10 ng/ml) for 4 h. (B & C) Results of protein band intensities are expressed as arbitrary units of phospho-ERK1/2 against total ERK1/2. Results are expressed as the mean ± SEM (n = 3) and significant difference from the respective IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, *p<0.05; ** p<0.01; *** p<0.001.

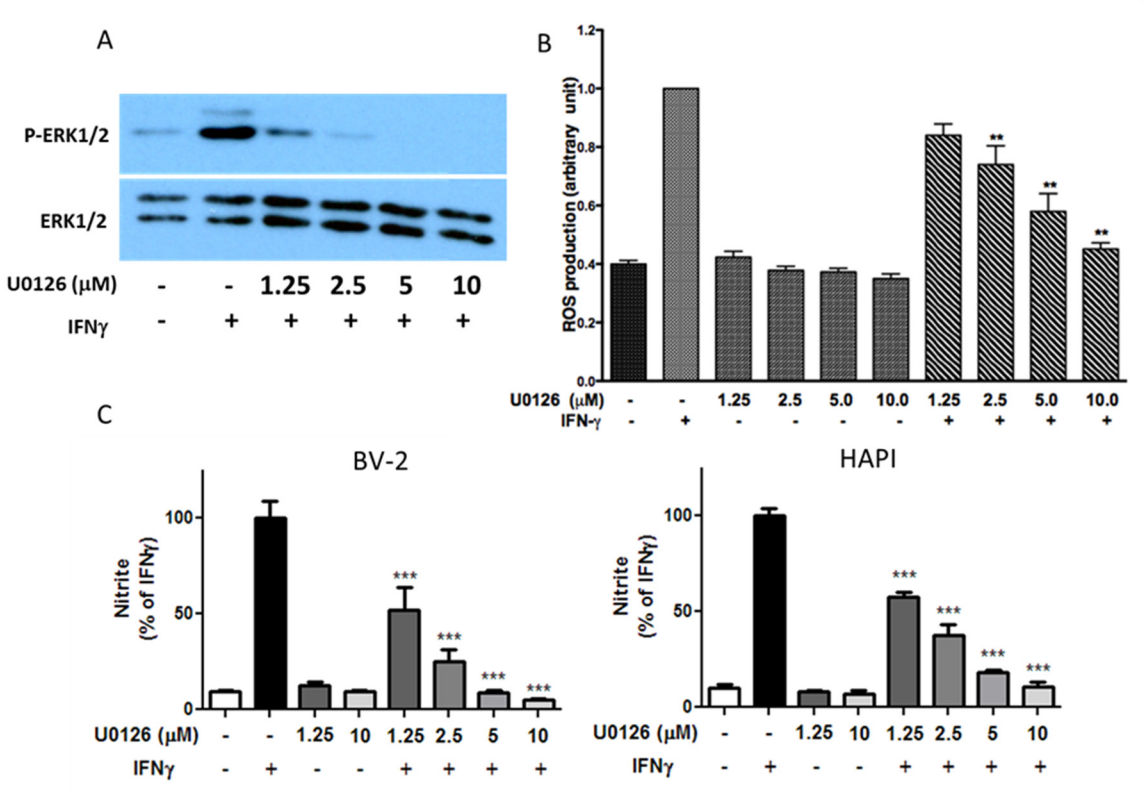

Figure 2.12

The role of ERK1/2 activation in IFNγ-induced ROS and NO production in BV-2 and HAPI microglial cells. Cells were treated with IFNγ (10 ng/ml) with or without the MEK1/2 inhibitor, U0126 (1.25 - 10 μM). (A) Representative Western blot demonstrating U0126 inhibiting phosphorylation of ERK1/2 dose-dependently 4 h after IFNγ treatment. (B) For ROS production, BV-2 cells were pretreated with different concentrations of U0126 for 1 h prior to stimulation with IFNγ for 12 h. (C) For NO production, both BV-2 and HAPI cells were pretreated with different concentrations of U0126 for 1 h prior to stimulation with IFNγ for 16 h. Results are expressed as the mean ± SEM (n = 3), and significant difference from the respective IFNγ stimulated group was determined by one-way ANOVA followed by Dunnett's tests, ** $p<0.01$; *** $p<0.001$.

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CHAPTER 3

CYTOSOLIC PHOSPHOLIPASE A2 PLAYS A CRUCIAL ROLE IN ROS/NO SIGNALING DURING MICROGLIAL ACTIVATION THROUGH THE LIPOXYGENASE PATHWAY

ABSTRACT

Background

Oxidative stress and inflammation are important factors contributing to the pathophysiology of numerous neurological disorders, including Alzheimer's disease, Parkinson's disease, acute stroke, and infections of the brain. There is well-established evidence that pro-inflammatory cytokines, glutamate, as well as reactive oxygen species (ROS) and nitric oxide (NO) are produced upon microglia activation, and these are important factors contributing to inflammatory responses and cytotoxic damage to surrounding neurons and neighboring cells. Microglial cells express relatively high levels of cytosolic phospholipase A_2 ($cPLA_2$), an enzyme known to regulate membrane phospholipid homeostasis and release of arachidonic acid (AA) for synthesis of eicosanoids. The goal for this study is to elucidate the role of $cPLA_2$IV in mediating the oxidative and inflammatory responses in microglial cells.

Methods

Experiments involved primary microglia cells isolated from transgenic mice deficient in $cPLA_2\alpha$ or $iPLA_2\beta$, as well as murine immortalized BV-2 microglial cells.

Inhibitors of $cPLA_2$/$iPLA_2$/cyclooxygenase (COX)/lipoxygenase (LOX) were used in BV-2 microglial cell-line. siRNA transfection was employed to knockdown $cPLA_2$ expression in BV-2 cells. Griess reaction protocol was used to determine NO concentration, and CM-H2DCF-DA was used to detect ROS production in primary microglia and BV-2 cells. WST-1 assay was used to assess cell viability. Western blotting was used to assess protein expression levels. Immunocytochemical staining for phalloidin against F-actin was used to demonstrate cell morphology.

Results

In both primary and BV-2 microglial cells, stimulation with lipopolysaccharide (LPS) or interferon gamma (IFNγ) resulted in a time-dependent increase in phosphorylation of $cPLA_2$ together with ERK1/2. In BV-2 cells, LPS- and IFNγ-induced ROS and NO production was inhibited by AACOCF3, pyrrophenone as well as RNA interference, but not BEL, suggesting a link between $cPLA_2$, and not $iPLA_2$, on LPS/IFNγ-induced nitrosative and oxidative stress in microglial cells. Primary microglial cells isolated from $cPLA_2\alpha$ deficient mice generated significantly less NO and ROS as compared with the wild type mice. Microglia isolated from $iPLA_2\beta$ deficient mice did not show a decrease in LPS-induced NO and ROS production. LPS/IFNγ induced morphological changes in primary microglia and these changes were mitigated by AACOCF3. Interestingly, despite that LPS and IFNγ induced increase in phospho-$cPLA_2$ and prostaglandin E2 (PGE2) release, LPS- and IFNγ-induced NO and ROS production were not altered by the COX1/2 inhibitor, but was suppressed by the LOX-12 and -15 inhibitors instead.

Conclusion

In summary, results in this study demonstrated the role of $cPLA_2$ in microglial activation with metabolic links to oxidative and inflammatory responses, and this was in part regulated by the AA metabolic pathways, namely the LOXs. Further studies with targeted inhibition of $cPLA_2$/LOX in microglia during neuroinflammatory conditions can be valuable to investigate the therapeutic potential in ameliorating neurological disease pathology.

INTRODUCTION

Neuroinflammation plays a major role in the progression of neurodegenerative diseases including Alzheimer's disease, Parkinson's diseases, cerebrovascular disease, and infectious HIV encephalopathy. Microglial cells, the resident innate immune cells in the central nervous system (CNS), are known to exert multiple physiologic functions in the brain, including anchoring CNS innate immune response through phagocytosis of foreign pathogens, removing cellular breakdown products, stimulating tissue repair processes, and maintaining tissue homeostasis (Aguzzi et al., 2013). Activation of microglial cells can also exert significant impact on the propagation of inflammatory responses (Block and Hong, 2005; Block et al., 2007). For instance, activated microglia were shown *in vivo* to contribute to expansion of infarct after focal cerebral ischemia (Mabuchi et al., 2000), and inhibition of microglial activation was proven a to be viable strategy to prevent inflammatory neuronal death *in vitro* (Neher et al., 2011). Recent

studies had place much focus on the differential functions of polarized M1/M2 microglial cells after activation. While much research is currently underway to distinguish the biochemical and functional properties of each phenotype, most tend to agree that M1 microglia are more cytotoxic and persist during the disease effector stage, whereas M2 microglia are more neuroprotective and predominate during the repair stage (Franco and Fernandez-Suarez, 2015; Hu et al., 2015; Tang and Le, 2015). The discovery of functional differences and delineation of time course of microglia polarization has generated interest in ways to limit M1 activation and stimulate M2 transformation in order to ameliorate outcomes of neurological diseases, including experimental stroke and traumatic brain injury (Hu et al., 2012; Wang et al., 2013; Pan et al., 2015; Wang et al., 2015; Xia et al., 2015).

Biochemically, M1 microglial activation is associated with the release of ROS, NO, glutamate, cytokines (such as TNFα), phospholipases, matrix metalloproteases, and other pro-inflammatory factors contributing to the progressive neuronal damage observed in many neurodegenerative disorders (Takeuchi et al., 2006; Brown, 2007; Chhor et al., 2013). Therefore, suppressing or limiting microglial activation can have beneficial effects for preventing neuroinflammation and neurodegeneration. Microglia *in vitro* can be activated with a variety of agents, such as proinflammatory cytokines (TNFα, IL-1β, IFNγ), lipopolysaccharides (LPS), and oligomeric beta amyloid (Aβ) (Hanisch, 2002). Studies including those from our laboratory have demonstrated that microglia activation by proinflammatory cytokines and LPS causes induction of iNOS and activation of NADPH oxidase, leading to increased oxidative/nitrosative stress (Sheng et al., 2011).

Phospholipase A_2s (PLA_2s) are groups of enzymes that hydrolyze the fatty acids from the *sn*-2 position of membrane phospholipids. Among the PLA_2s identified, $cPLA_2$ and $iPLA_2$ are the constitutively active PLA_2s that serve as important mediators for the release of polyunsaturated fatty acids, including arachidonic acid (AA) and docosahexaenoic acid from membrane phospholipids (Burke and Dennis, 2009; Leslie, 2015). Multiple studies have demonstrated group IV $PLA_2\alpha$ ($cPLA_2\alpha$) to be the major PLA_2 responsible for the release of AA and to play an essential role in inflammation. Transgenic mice lacking $cPLA_2\alpha$ have been shown to display significantly reduced deleterious phenotypes in inflammatory diseases, such as ischemic brain injury, anaphylaxis, arthritis, alcoholism, and acute lung injury (Bonventre et al., 1997; Nagase et al., 2000; Nagase et al., 2002; Raichel et al., 2008; Kishimoto et al., 2010; Tai et al., 2010; Tajuddin et al., 2014). More recent *in vivo* studies demonstrated ability for pharmacological inhibitors of $cPLA_2$ to ameliorate ischemic stroke, experimental autoimmune encephalitis, and spinal cord injury (Vana et al., 2011; Zhang et al., 2012; Liu et al., 2014). Although $cPLA_2$ activation in brain is associated with oxidative stress, neuronal excitation, and neuroinflammation (Shelat et al., 2008), little is known about mechanism(s) for its activation in microglial cells (Chuang et al., 2014). Previous studies demonstrated protective effects of $cPLA_2$ inhibition against microglia-induced white matter damage *in vivo* and oligodendrocyte cell death *in vitro*, suggesting the role of this enzyme as a potential target to suppress microglia-induced secondary damage in the central nervous system (Vana et al., 2011). However, the mode of action of $cPLA_2$ and its link to the inflammatory responses in microglial cells have not been elucidated in detail.

In this study, we isolated primary microglial cells from $cPLA_2$ and $iPLA_2$ KO mice to demonstrate the role of $cPLA_2$ (and not $iPLA_2$) in mediating oxidative and inflammatory responses from LPS and IFNγ stimulation. In addition, we further suggested a mechanism that links $cPLA_2$ mediated eicosanoid production with downstream ROS and NO generation in BV-2 microglial cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and 0.25% (w/v) trypsin/EDTA were obtained from GIBCO (Gaithersburg, MD). Endotoxin-free fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA). Lipopolysaccharide (LPS) (rough strains) from Escherichia coli F583 (Rd mutant) was purchased from Sigma-Aldrich (St. Louis, MO). Interferon-γ (IFNγ) was purchased from R & D Systems (Minneapolis, MN). Pharmacological inhibitors used include the following: U0126, SB202190, and SP600125 were from Cell Signaling (Beverly, MA). Arachidonyl trifluoromethyl ketone (AACOCF3), Pyrrophenone, racemic bromoenol lactone (BEL), nordihydroguaiaretic acid (NDGA), Ibuprofen, Zileuton, and PD146176 were from Cayman Chemical (Ann Arbor, MI). NCTT-956 was from Sigma-Aldrich (St. Louis, MO). RNA interference Lipofectamine RNAiMAX Transfection Reagent was from Life Technology (Carlsbad, CA). siRNA against $cPLA_2$ Mm_Pla2g4a_8 FlexiTube siRNA (NM_008869) and AllStars Negative Control siRNA were purchased from Qiagen (Hilden, Germany). Antibodies used for Western blots include: goat anti-rabbit

IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase, anti-cPLA$_2$ rabbit polyclonal, anti-iNOS rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-β-actin peroxidase (Sigma-Aldrich, St. Louis, MO); rabbit polyclonal anti-p-cPLA$_2$, rabbit polyclonal anti-ERK1/2, and mouse monoclonal anti-phospho-ERK1/2 antibodies (Cell Signaling, Beverly, MA). An affinity-purified antibody directed against an iPLA$_2$β peptide corresponding to residues 277-295 was a gift of Drs. Chris Jenkins and Richard Gross (Washington University School of Medicine, St. Louis, MO) (Mancuso et al., 2003). For immunocytochemical staining, rabbit anti-Ionized calcium-binding adapter molecule 1 (Iba-1) antibodies (019-19741) were purchased from Wako BioProducts (Richmond, VA), Alexa Fluor 488® phalloidin from Life Technologies (Carlsbad, CA), and 4',6-diamidino-2-phenylindole (DAPI) from Roche Molecular Chemicals (Basel, Switzerland). For ROS detection, CM-H2DCF-DA (DCF) was purchased from Invitrogen, Inc. (Eugene, OR). WST-1 assay was purchased from Clontech (Mountain View, CA). PGE2 EIA Kit was purchased from Cayman Chemicals (Ann Arbor, MI).

cPLA$_2$ transgenic animal breeding and genotyping

All animal care and experimental protocols were carried out in accordance with NIH guidelines and with permission from the University of Missouri Animal Care and Use Committee (protocol #6728). Pairs of C57Bl/6 male and female heterozygous cPLA$_2$+/− mice were kindly provided by Dr Joseph V. Bonventre (Harvard Medical School, Boston, MA) and colony was expanded at the University of Missouri for more than 5 generations prior to start of the experiments. Wild-type cPLA$_2$+/+ and

homozygous knockout cPLA$_2$-/- mice used in the experiments were generated by crossing male and female heterozygous cPLA$_2$+/- mice, and genotyping of litters was done between postnatal day 3~6 by polymerase chain reaction (PCR) as previously described (Bonventre et al., 1997).

iPLA$_2$ transgenic animal breeding and genotyping

iPLA$_2$β-KO mice were housed and cared for in animal facilities administered through the Washington University Division of Comparative Medicine, and animal procedures were performed according to a protocol approved by the Washington University Animal Studies Committee. iPLA$_2$β-KO mice were previously generated by insertion of the neomycin resistance gene into exon 9 of the mouse iPLA$_2$β (*Pla2g6*) gene by homologous recombination (Bao et al., 2004; Malik et al., 2008). KO and WT mice were generated by mating heterozygous mice, and their genotype was determined by a polymerase chain reaction (PCR) assay. The primers used for PCR genotyping were WT F1 (TTACCTCCGCTTCTCGTCCCTCATGGAGCT), Neo F1 (GGGAACTTCCTGACTAGGGGAGGAGTAGAA), and WT R1 (TCTGTTTCTCTAGAGACCCATGGGGCCTTG), which when combined in a single PCR reaction generate a 158 bp band for the WT allele and a 254 bp band for the KO allele.

Primary microglia isolation

Preparations of primary microglial cells with postnatal day 7-10 C57Bl/6 pups were accomplished with the Miltenyi Biotec MACS cell separation system (Bergisch

Gladbach, Germany). Briefly, brains from the genotyped pups were dissected and meninges removed. Tissues were dissociated using the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) with the gentleMACS dissociator. Prior to isolation, cell concentration in the suspension was counted and roughly 10^5 cells were collected for flow cytometry analysis by the Cellular Immunology Core in the University of Missouri. Microglia were isolated from the single-cell suspension using the Magnetic activated cell sorting (MACS) Technology with anti-cluster of differentiation molecule 11b (CD11b) (Microglia) MicroBeads (Miltenyi Biotec) in combination with an OctoMACS Separator with slight modifications to the manufacturer's instructions. The number of cells post-isolation was counted, and roughly 10^5 cells were collected for post-isolation flow cytometry analysis, and the remaining cells were plated at a density of $5x10^5/cm^2$. Plated cells were cultured in DMEM supplemented with 10% FBS containing 100 units/mL penicillin and 100 µg/mL streptomycin, and maintained in 5% CO_2 incubator at 37°C. Culture medium was replaced every 3-5 days. Primary cell cultures were used between days-in-vitro (DIV) 5-7.

Immortalized microglial BV-2 cell culture

The murine BV-2 cell line was generated by infecting primary microglia cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2) (Blasi et al., 1990). These cells were obtained as a gift from Dr. R. Donato (Adami et al., 2001) and prepared as previously described (Shen et al., 2005; Sheng et al., 2011). Briefly, cells were cultured in DMEM (high glucose) supplemented with 10% FBS containing 100 units/mL penicillin and 100 µg/mL streptomycin, and maintained in 5% CO_2 incubator at 37°C.

For subculture, cells were removed from the culture flask by gentle scraping, re-suspended in the culture medium, and sub-cultured in 6/96-well plates for experiments. Cell condition and morphology were assessed by using a phase contrast Nikon DIAPHOT 300 microscope attached with a CCD cool camera, and MagnaFire2.1C software was used for image capture and processing. Representative bright field pictures were obtained using a 20x objective lens.

Flow cytometry for microglial cell purity analysis

During primary microglia isolation, after tissue dissociation and cell number was determined, roughly 10^5 cells were collected, resuspended in 100μL buffer and incubated with 10 μL CD11b-FITC antibodies (Miltenyi) for 10 mins in 4°C. Cells were then washed and resuspended in 100 μL fresh buffer for flow cytometry analysis. Similarly, after microglia isolating by CD11b cell sorting, 10^5 cells were collected and labeled with cd11b-FITC for flow cytometry analysis. Flow cytometry analysis was performed using the BD FACScan under the FITC protocol by the Cellular Immunology Core in the University of Missouri.

$cPLA_2$ RNA interference knockdown in BV-2 cells

BV-2 cells were seeded in 96- and 24-well plates with antibiotics-free DMEM containing 5% FBS for 24 h. When cell density reached roughly 70-80%, they were transfected with either AllStars negative control siRNA (Qiagen), or $cPLA_2$ siRNA (NM_008869, Qiagen) (final concentration of 40 nM) using the RNAiMAX transfection reagent (Invitrogen) in mixture of Opti-MEM and DMEM mediums for 48 h prior to

being used for experiments, according to the manufacturer's instructions. $cPLA_2$ knockdown was evaluated by Western blot for protein expression of total $cPLA_2$ normalized against β-actin.

Cell viability assay protocol

The WST-1 protocol was used for assessment of cell viability. Briefly, after reaching 80-90% confluence, cells in 96-well plates were serum starved for 4h, followed by incubation with inhibitors for 16h. After treatment, cell viability was determined by adding 10 μL of the WST-1 reagent (Roche Applied Science, Germany) into each well. After gentle shaking, cells were incubated for 1 h at 37°C and absorbance was read at 450 nm (with reference wavelength at 650 nm).

NO determination

NO released from BV-2 cells was converted to nitrite in the culture medium. NO concentration was measured using the Griess reagent protocol as described previously (Sheng et al., 2011). In brief, BV-2 cells in a 96-well plate were serum-starved in phenol red-free DMEM for 3h, followed by incubation with designated inhibitors for 1 h. Cell were then incubated with IFNγ or LPS at 37˚C for 16h. Alternatively, primary microglia were stimulated with IFNγ or LPS at 37˚C for 24/48 h. Aliquots of medium (50 μL) were incubated with 50μL of the reagent A [1% (w/v) sulfanilamide in 5% phosphoric acid, Sigma-Aldrich] for 10 minutes at room temperature covered in dark. This was followed by addition of 50 μL of reagent B [0.1%, w/v, N-1-napthylethylenediamine dihydrochloride, Sigma-Aldrich] for 10 mins at room temperature, protected from light,

and absorbance at 543 nm was measured using a microplate reader (Biotek Synergy 4, Winooski, VT). Serial dilutions of sodium nitrite (0-100 μM) were used to generate the nitrite standard curve.

ROS determination

ROS production in microglial cells was assessed with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA, or DCF in short). Primary microglia or BV-2 microglial cells were seeded in 96-well plate and grown until 90% confluent. Cells were serum-starved for 3 h, followed by pretreatment with inhibitors for 1h, prior to stimulation with LPS or IFNγ for 11 h. Alternatively, primary microglia were stimulated with IFNγ or LPS at 37˚C for 24/48 h. DCF (10 μM) was added to each well and incubated for 1h. The fluorescent intensity of DCF was measured with a microplate reader (excitation wavelength of 490 nm and emission wavelength of 520 nm). Fluorescent intensity was normalized against control wells for statistical analysis.

PGE2 ELISA protocol

PGE2 concentration in the cell conditioned medium was assessed with the PGE2 ELISA protocol (Cayman Chemicals). Briefly, 50 μL of conditioned medium from treated BV-2 cells in 96-well plates were incubated with 50 μL of PGE2 monoclonal antibody and 50 μL of PGE2 AChE tracer for 18 h at 4°C with plates covered with plastic film. Standard curve was generated with serial dilution of PGE2 EIA standard (Cayman No. 414014) in EIA buffer prepared according to Cayman's protocol. On day 2, 100 dtn of Ellman's Reagent was reconstituted with 20 mL of UltraPure water and 200 μL added

into each sample/standard well for development. Plates were placed on an orbital shaker for 60 mins prior to measuring for absorbance at 410 nm using a microplate reader. Concentration was calculated from the 4th degree polynomial curve generated from the standard wells in accordance to kit instruction.

Western blot analysis

Cell lysates were collected in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS. The extract was centrifuged at 10,000 × g for 15 min at 4°C and transferred to a clean tube to remove cell debris. Protein concentration was measured and normalized with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Depending on the target of interest, 5-10 μg of total protein was loaded in SDS-PAGE for electrophoresis. After electrophoresis, proteins were transferred to 0.45 μm nitrocellulose membranes. Membranes were incubated in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk for 1 h at room temperature. The blots were incubated at 4°C overnight with antibodies $cPLA_2$ (1:1000), phospho-$cPLA_2$ (1:1000), ERK1/2 (1:2000), phospho-ERK1/2 (1:1000), iNOS polyclonal (1:1000) and β-actin (1:50000). After repeated washing with 1X TBS-T, blots were incubated with goat anti-rabbit IgG-HRP (1:4000) or goat anti-mouse IgG-HRP (1:2000) for 1 h at room temperature. Immuno-labeling was detected by chemiluminescence ECL/WestPico/Femto and developed in X-ray film developer. Films were scanned and the optical density of bands was measured with the QuantityOne software (BioRad, Hercules, CA).

Immunocytochemistry staining

Immunocytochemistry staining was carried out as previously described by Chuang et al. (Chuang et al., 2013). Briefly, cells were cultured in 24-well plates containing round cover slips. After treated, cells were fixed in 4% paraformaldehyde for 15 min and then permeabilized with 0.1 % Triton X-100 in PBS for 30 min. Cells were incubated with 10% normal goat serum in 0.005% Triton X-100 in PBS for 60 min, then incubated overnight in 0.5% normal goat serum in 0.005% Triton X-100 in PBS containing primary antibodies. The next day, cells were incubated in 0.005% Triton X-100 in PBS containing secondary antibodies, goat-anti-rabbit Alexa fluor 488 (Jackson Immunoresearch) and goat-anti-mouse Alexa fluor 549 (Jackson Immunoresearch) for 60 min, followed by 1 unit of Alexa Fluor 488 phalloidin (Life Technologies)/well for 20 min (5uL of 6.6μM stock solution dissolved in methanol diluted in 200 μL of 0.005% Triton X-100 in PBS), and nuclear counterstaining with 1 μg/ml of 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Pierce) in PBS for 10 min. The coverslips were then mounted on fluoromount (Sigma-Aldrich) and sealed with nail polish. Fluorescence photomicrographs were captured using a Leica DMI 6000B fully automated epifluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Statistical analysis

Data were presented as means ± SEM. Results were analyzed either by one-way ANOVA followed by Dunnett's multiple comparison tests (V4.00; GraphPad Prism Software Inc., San Diego, CA). Statistical significance was considered for $p < 0.05$.

RESULTS

Stimulation of iNOS, p-ERK1/2 and p-cPLA$_2$ protein expression by LPS and IFNγ in primary and immortalized (BV-2) microglial cells.

In this study, we first characterized primary microglial cells isolated from 7-10 days postnatal c57bl/6 WT and cPLA$_2$ KO mouse brains using the Miltenyi Biotec MACS protocol. Flow cytometry analysis showed 14% of cells from the homogenized brain tissues expressed CD11b surface antigen (microglia) prior to cell sorting/isolation (Fig. 3.1A). After MACS isolation, more than 90% of isolated cells were CD11b positive, indicating relatively high purity from the isolation protocol (Fig. 3.1B). Immunocytochemistry staining with antibodies targeted against CD11b followed by fluorescent microscopy demonstrated staining in nearly all cells with morphology that resembled ramified microglial cells (Fig. 3.1C).

Our earlier studies have demonstrated the ability for BV-2 microglial cells to upregulate iNOS and produce NO after LPS or IFNγ stimulation individually, and proposed ERK1/2 as one of the central components in mediating this transcriptional process (Chuang et al., 2013; Jiang et al., 2014). In this study, a time course study was carried out to test induction of iNOS, p-ERK1/2 and p-cPLA$_2$ by LPS and IFNγ using primary cells and compare with BV-2 microglial cells. When primary microglial cells were stimulated with LPS (200 ng/ml), ERK1/2 was phosphorylated within an hour, and started to decline after 2 hours albeit remaining elevated up to 24 h compared with baseline (Fig. 3.2A). Following the increase in pERK1/2, LPS also induced an increase in cPLA$_2$ phosphorylation, with maximal expression occurring at 4 hours prior to a gradual

decline (Fig. 3.2A). On the other hand, iNOS expression was not observed until after 8 hours and was highest at 24 hours (Fig. 3.2A). A similar expression profile was observed when primary microglia were stimulated with IFNγ (20 ng/ml), although the sequence of events appeared to be delayed with ERK1/2 phosphorylation maximized at 4 hours, $cPLA_2$ phosphorylation maximized at 8 hours, and iNOS expression not observed until 16 hours post-stimulation (Fig. 3.2B). With IFNγ, p-$cPLA_2$ remained up-regulated at 24 hours post-stimulation. There were no significant changes in total ERK1/2 or $cPLA_2$ protein during the activation process. When the same conditions were subjected to BV-2 cells, matching trends were observed among the proteins of interest (Fig. 3.2C-D), with exception that LPS stimulation of p-ERK1/2 and p-$cPLA_2$ appeared to be stronger than the primary microglial cells and remained up-regulated at 24 hours. These results suggest the use of BV-2 cells as a justified model system to investigate biochemical profiles of our pathway of interest during microglial activation.

$cPLA_2$ phosphorylation is regulated by phospho-ERK1/2

Among multiple sites, Ser505 residue is the site phosphorylated by MAPKs in $cPLA_2$ (Leslie, 1997). In this study, we examined the effects of MAPK inhibitors, U0126 (MEK1/2-ERK1/2 inhibitor), SB202190 (p38 MAPK inhibitor), and SP600125 (JNK inhibitor) on $cPLA_2$ phosphorylaton after stimulation by LPS, at 2 hours post-stimulation, and by IFNγ at 8 hours post-stimulation in BV-2 cells. Results demonstrated that among the inhibitors tested, U0126 inhibited Ser505 phosphorylation in a dose-dependent manner whereas SB202190 and SP600125 were not effective (Fig. 3.3A and C). Similar results were observed with IFNγ (Fig. 3.3B and D). These results are consistent with the

notion that phospho-ERK1/2 serves as the primary regulator of $cPLA_2$ phosphorylation in microglia cells.

LPS and IFNγ induced iNOS expression and NO production are significantly decreased in microglia deficient in $cPLA_2$

In this study, primary microglial cells isolated from WT and $cPLA_2$ -/- homozygous KO mice brains were used to test for their ability to induce iNOS expression and NO production upon stimulation with LPS or IFNγ. As expected, $cPLA_2$ expression was blunted in microglia isolated from the $cPLA_2$ KO brain as compared to the WT brain (Fig. 3.4A). Under the same conditions, LPS- induced iNOS expression and NO production were significantly decreased in microglia isolated from the $cPLA_2$ KO brain as compared to the WT brain (Fig 3.4A, B, and D). Similarly, IFNγ-induced iNOS expression and NO production were also significantly decreased in microglial cells from the $cPLA_2$ KO brains (Fig. 3.4A, C and D). The concentration of LPS and IFNγ used in our experiment was shown not to cause significant cell death at the given time points (Fig. 3.5).

LPS and IFNγ-induced ROS from WT and $cPLA_2$ KO microglia

Our earlier study demonstrated the temporal profile and mechanism for LPS and IFNγ to induce ROS production in BV-2 microglial cells (Chuang et al., 2013). In this study, we attempted to compare ROS production between primary microglia isolated from WT and $cPLA_2$ KO brains. In WT primary microglial cells, ROS production was maximally increased after stimulation with 200 ng/ml LPS and continued to increase for

48 hours (Fig. 3.6A). ROS induced by IFNγ was not significantly increased at 24 hours, but continued to rise at 48 hours (Fig. 3.6B). Under the same conditions and stimulus concentrations, neither LPS nor IFNγ managed to cause significant increase in ROS production in primary microglia isolated from $cPLA_2$ KO brain (Fig. 3.6C-D).

Pharmacological inhibition and siRNA of $cPLA_2$ result in suppression of LPS- and IFNγ-induced NO production in BV-2 cells

Based on the above data suggesting a link between $cPLA_2$ and LPS/IFNγ-induced NO production, we further tested whether inhibition of $cPLA_2$ by pharmacological inhibitors and by siRNA knockdown may alter the ability for LPS and IFNγ to stimulate NO in BV-2 microglial cells. In this study, two pharmacological inhibitors were used: AACOCF3, a non-specific PLA_2 inhibitor known to suppress activity of both $cPLA_2$ and $iPLA_2$, and pyrrophenone, a specific $cPLA_2$ inhibitor. As shown in Fig. 3.7A-D, both inhibitors showed dose-dependent suppression of NO generation 16h after LPS and IFNγ stimulation. The doses of AACOCF3 and pyrrophenone used in this study were verified to not cause toxicity in the culture system using the WST-1 assay while aiming for reasonable level for maximum effect (data not shown).

RNA interference was further employed to ensure the inhibition observed above did not result from non-specific pharmacological effects. Using the $cPLA_2$ siRNA (NM_008869, Qiagen) and RNAiMAX transfection reagent, we were able to knockdown $cPLA_2$ by 70-80%, based on protein expression by Western blot (Fig. 3.8). Under this

condition, NO production was significantly suppressed in knockdown cultures as compared with control cultures (Fig. 3.7E-F).

Pharmacological inhibition and siRNA of cPLA$_2$ result in significant suppression of LPS- and IFNγ-induced ROS production in BV-2 cells

Our previous study demonstrated that ROS production from NADPH oxidase activation plays a major role in microglia activation and it precedes iNOS induction and NO production (Chuang et al., 2013). In this study, cPLA$_2$ inhibitors and siRNA were used to test the link between cPLA$_2$ and LPS or IFNγ induced ROS production in BV-2 cells. As shown in Fig. 3.9A-D, both AACOCF3 and pyrrophenone dose-dependently inhibited LPS- or IFNγ-induced ROS production when measured at 12 hours post-stimulation. Similarly, siRNA also significantly diminished ROS induction by the two stimuli (Fig. 3.9E-F).

cPLA$_2$ inhibition prevents morphological changes associated with activation in primary microglia

In order to visualize the morphological changes of microglia under activation by LPS or IFNγ, primary microglia were cultured in coverslips, followed by fixation and immunostaining with Iba-1 (red), a marker for microglial cells, and phalloidin (green) for actin filaments. Undisturbed microglia cells were uniform in size, and displayed small round cytoplasm with an off-centered nucleus, resembling the resting ramified phenotype (Fig. 3.10A-B). At 24 hours after LPS stimulation, there was obvious expansion of cytoplasmic space with significant formation of filopodia (Fig. 3.10C-D). Cells treated

with IFNγ appeared to show higher Iba1 staining with cytoplasm more spread out like a fried egg. Some also showed extensive budding around the periphery (Fig. 3.10E-F). There were also increased mitotic events as evident by cells with di-nuclei in both types of stimulation. Interestingly, microglia pretreated with 5μM of AACOCF3 for one hour prior to LPS or IFNγ stimulation showed preservation of morphology closely resembling unstimulated ramified microglia in the control plates (Fig. 3.10G-J).

Ca^{2+}-independent PLA_2 does not alter LPS- and IFNγ-induced iNOS/NO/ROS production

$cPLA_2$ and $iPLA_2$ are both constitutively expressed in most cell types, and are both possible contributors to AA production along with its downstream cascade. While we had established $cPLA_2$ to play crucial role in microglia activation, it is also important to investigate whether $iPLA_2$ may also play a role in this process. Using the Miltenyi Biotec MACS cell separation system, primary microglial cells were isolated from WT and $iPLA_2$ KO brains. As shown in Fig. 3.11A, expression of $iPLA_2$ was not observed in the $iPLA_2$ KO brains, and stimulation with LPS did not result in a significant difference in iNOS expression and NO production between WT and KO microglia (Fig. 3.11B-C).

To further verify the results, we also tested whether selective $iPLA_2$ inhibitor BEL (racemic) may have an effect on LPS- and IFNγ-induced NO and ROS production in BV-2 cells. As shown in Fig. 3.11D-G, results indicated that BEL did not significantly affect the amount of NO or ROS produced by either stimulus.

cPLA$_2$-dependent induction of NO or ROS in microglia does not go through COX1/2

cPLA$_2$ is responsible for AA production, and downstream eicosanoid production. AA can be converted by COX1/2 into prostaglandin H2 which is further metabolized to prostaglandins, prostacyclin, and thromboxanes. Alternatively, AA can also go through the lipoxygenase (LOX) pathway to generate 5/12/15-hydroperoxyicosatetraemoic acid (HPETE). COX1/2 and prostaglandins have always been implicated in inflammatory processes and COX1/2 remains a popular target of anti-inflammatory therapy by non-steroidal anti-inflammatory drugs (NSAIDs). In the following experiments, we tested the involvement of COX1/2 in NO/ROS production in BV-2 cells. Using the ELISA protocol, we measured the concentration of PGE2 in a conditioned medium of BV-2 microglial cell cultures after stimulation with LPS or IFNγ. We further investigated the effect of ibuprofen, a non-selective reversible COX1/2 inhibitor, to inhibit PGE2 production. Results showed a dose-dependent inhibition of LPS- and IFNγ-induced PGE2 by ibuprofen (Fig. 3.12A-B). We further tested whether ibuprofen could inhibit LPS and IFNγ-induced NO and ROS in BV-2 cells. Interestingly, ibuprofen did not exert inhibitory effects on either LPS- or IFNγ-induced NO and ROS production (Fig. 3.12C-F).

LOX inhibition significantly suppresses cPLA$_2$-dependent microglial induction of ROS and NO

cPLA$_2$-induced AA release can be metabolized by either COX or LOX. Since the above results indicated that COX played a minimal role in ROS/NO production, experiments were directed to test whether the LOX pathways may mediate LPS- and

IFNγ-induced ROS and NO production. The LOX products have been shown to provide an important role in mediating the downstream inflammatory leukotrienes in neurodegenerative conditions and infectious processes (Phillis et al., 2006). When BV-2 cells were pretreated with NDGA, a non-selective LOX inhibitor, NO and ROS production was significantly suppressed in a dose-dependent manner (Fig. 3.13A-D).

Among the lipoxygenases, LOX-5, LOX-12, and LOX-15 are the most studied and are responsible for the generation of 5-HPETE, 12-HPETE, and 15-HPETE, respectively. To further investigate which LOX and its subsequent products were responsible for LPS- or IFNγ-induced ROS and NO production in microglia, we incorporated the use of Zileuton, NCTT-956, and PD146176, previously described selective inhibitors for LOX-5, LOX-12, and LOX-15, respectively (Carter et al., 1991; Sendobry et al., 1997; Kenyon et al., 2011). While Zileuton at varying concentrations did not seem to affect production of either NO or ROS production by BV-2 cells after LPS stimulation (Fig. 3.14 A-B), both NCTT-956 and PD146176 inhibited ROS/NO production in a concentration dependent manner (Fig. 3.14C-F). Similar results were seen when BV-2 cells were stimulated by IFNγ (Fig. 3.15A-F). These results thus provided information that LPS- and IFNγ-induced ROS and NO in microglial cells may be regulated by LOX-12/15 and not LOX-5.

DISCUSSION

$cPLA_2$ plays a significant role in microglial activation

$cPLA_2$ has been shown to play a significant role in mediating oxidative/nitrosative and inflammatory responses in neurons, astrocytes and other cells (Sun et al., 2012; Sun et al., 2014), but less attention has been paid to microglia. The findings of this study clearly demonstrate the involvement of $cPLA_2$ in LPS- and IFNγ-induced ROS and NO production in microglial cultures. To our knowledge, this is the first study to use primary microglia prepared from $cPLA_2$ and $iPLA_2$ knockout mice to provide new evidence for the significant role of $cPLA_2$ in microglial activation.

The comparison between $cPLA_2$ knockout and wild type primary microglia cells showed that $cPLA_2$ not only plays a crucial role in activating the oxidative/inflammatory pathway, leading to generation and release of ROS/NO, but also to the overall morphological transformation of microglial cells after endotoxin/cytokine stimulation. To ensure that the findings are not a result of alternative mechanisms from long-term functional compensation in response to $cPLA_2$ knockout, the same conclusion was reached using pharmacological inhibition, with AACOCF3 (non-specific) and pyrrophenone (specific), and RNA interference knockdown in BV-2 cells. Other studies using LPS but not IFNγ as a stimulator support this conclusion in rat primary microglia (Vana et al., 2011) and BV-2 cells (Ribeiro et al., 2013). On the other hand, while $iPLA_2$ is also constitutively expressed in microglia and a target of AACOCF3, results here with $iPLA_2$ KO and pharmacological inhibition with BEL showed that this PLA_2 has little role in mediating ROS and NO production in the BV-2 microglial cells. This result is in slight

contrast to the prior study by Strokin et al., who suggested that $iPLA_2$ also contributes to the proinflammatory responses in LPS-treated astrocytes via Ca^{2+} signaling (Strokin et al., 2011). This difference may well be due to use of different cell types, i.e., microglia versus astrocytes.

The ERK1/2-$cPLA_2$-ROS-iNOS axis in microglial activation

$cPLA_2$ is known to have multiple active serine residues susceptible to phosphorylation. Among these serine residues, Ser505 was identified to be phosphorylated by MAPK and served as an important regulator for $cPLA_2$ activity and subsequent AA release (Leslie, 1997; Chuang et al., 2014). In the study by Pavicevic with vascular smooth muscle cells, phosphorylation of Ser515 by CaMKII was shown to precede Ser505 phosphorylation and phosphorylation of both Ser515 and 505 sites is required for activation of this enzyme (Pavicevic et al., 2008). In primary neurons in culture, stimulation with ionotropic glutamate receptor agonists such as NMDA resulted in ROS production through NADPH oxidase, and rapid activation of ERK1/2 and $cPLA_2$ (Shelat et al., 2008). In this study, we demonstrated that LPS and IFNγ each mediated a time-dependent increase in phospho-ERK1/2 and $cPLA_2$ in both primary and BV-2 microglial cells. In both conditions, the time for increase in p-ERK1/2 preceded that for p-$cPLA_2$. The relationship between p-ERK1/2 and $cPLA_2$ was further confirmed by U0126, the MEK1/2-ERK1/2 inhibitor, which readily abrogated phosphorylation of $cPLA_2$.

NADPH oxidase in microglial cells has been shown to play significant role in neurodegenerative diseases, such as alcohol induced neurodegeneration (Qin and Crews, 2012), Alzheimer's disease (Shimohama et al., 2000), and Parkinson's disease (Wu et al., 2003). A previous study from our laboratory has demonstrated the production of ROS from NADPH oxidase to be upstream of NO production in BV-2 microglia cells. Our studies further demonstrated that in BV-2 microglial cells, LPS and IFNγ can individually stimulate ROS and iNOS/NO through phosphorylation of ERK1/2 (Chuang et al., 2013; Jiang et al., 2014). A study by Ribeiro et al. (2013) also demonstrated effects of cannabinoid receptor agonists and antagonists, suppressing LPS-induced microglia activation via ERK1/2, $cPLA_2$ and NF-kB. These results, as well as ours, placed LPS and IFNγ activation of ERK1/2 and $cPLA_2$ upstream of the NF-kB transcriptional pathway. In rat microglial cells, Szaingurten-Solodkin observed a link between $cPLA_2$ in NADPH oxidase and iNOS activated by aggregated Abeta1-42, a toxic peptide cleaved from the amyloid precursor protein (Szaingurten-Solodkin et al., 2009). In their study, it was proposed that $cPLA_2$ regulated NADPH oxidase activity, which in turn caused upregulation of $cPLA_2$, COX1/2, and iNOS through an NF-kB-dependent mechanism. Taken together, our results with $cPLA_2$ inhibitors as well as siRNA knockdown, well demonstrated the role of $cPLA_2$ in mediating ROS and NO production upon stimulation by LPS and IFNγ. Our results with primary microglia isolated from $cPLA_2$ KO brains further validated the link between $cPLA_2$ on ROS and NO production in these cells.

Role of arachidonic acid and LOX in microglial activation

Earlier studies had linked $cPLA_2$ or its downstream metabolites (i.e. AA or lysophospholipids) with ROS production from NADPH oxidase, although the exact mechanism remains to be investigated (Dana et al., 1998; Shmelzer et al., 2003). In macrophages, there is evidence that $cPLA_2$ can interact directly with NADPH oxidase subunits, namely p47phox and p67phox, which facilitate translocation of these subunits to membranes to form the active NADPH oxidase complex (Zhao et al., 2002; Shmelzer et al., 2003). Alternatively, downstream products of AA were proposed to be involved in ROS production from NADPH oxidase (Levy et al., 2000). Activation of $cPLA_2$ and subsequent release of AA has been shown in the production of an array of eicosanoids, including the production of prostaglandins and leukotrienes through activation of COX and LOX. However, the extent for this action is cell dependent (Phillis et al., 2006). A number of studies, including those from our own, have demonstrated the increase in PGE2 production upon stimulation with LPS and IFNγ in astroglial cells (Xu et al., 2002; Xu et al., 2003). In the present study with microglial cells, we showed that while the COX1/2 inhibitor effectively inhibited LPS- and IFNγ-induced PGE2 production, this condition was not linked to the suppression of ROS and NO production by LPS and IFNγ.

While the action of COX1/2 is well-established in peripheral inflammation and a popular target for non-steroidal anti-inflammatory drugs (NSAIDs), its role in neuroinflammation is not well understood. Aspirin is used after acute stroke not for anti-inflammatory effect, but rather for secondary prevention of atherosclerosis due to its

antiplatelet properties through inhibition of prostaglandin and subsequent thromboxane A_2 (Patrono, 1994; Antithrombotic Trialists, 2002). Similarly, numerous recent large-scale double-blind placebo-controlled clinical trials have not found beneficial effects of COX-1/2 inhibition in the treatment of neurological diseases where neuroinflammation is proposed to be involved, such as Alzheimer's disease or depression (Trepanier and Milgram, 2010; Maes, 2012). In agreement with our study, Minghetti and collegues have also reported that microglial cell activation increased TNFα and COX-1/2, but this condition did not contribute to ROS/NO production (Minghetti, 2004, 2007).

On the other hand, recent studies have generated growing recognition of LOX in mediating inflammation, and some have implicated its role in neuroinflammation. Lipoxygenases are known to mediate the pathophysiology of numerous inflammatory diseases, including asthma, immune disorders and cancer. Parallel to the action of COX1/2 for the biosynthesis of prostaglandins from AA, lipoxygenases mediate the biosynthesis of leukotrienes and eoxins from AA, all of which are eicosanoids that play a significant role in inflammation and immune function (Phillis et al., 2006). Of note, the ability for LOX-15 to generate eoxin has been identified as a novel pathway of inflammatory responses in mast cells and eosinophils (Feltenmark et al., 2008), and as a promising novel target against asthma (Sachs-Olsen et al., 2010). Genetic ablation of LOX-12/15, but not LOX-5, was shown to protect against denervation-induced muscle atrophy (Bhattacharya et al., 2014). In the central nervous system, LOX-12/15 was shown to have increased expression in oligodendrocytes and microglia in periventricular leukomalacia (Haynes and van Leyen, 2013), and disease phenotype was ameliorated by

absence of LOX-12/15 in animal models of Alzheimer's disease (Yang et al., 2010). The LOX-5 pathway has also been associated with Alzheimer's disease and other neurodegenerative conditions (Joshi and Pratico, 2014). Although the mechanism of how LOX causes microglial activation and neuroinflammation remains to be elucidated, our results provided evidence suggesting that LOX activation in microglial cells plays a crucial role leading to induction of ROS and NO. To our knowledge, this is the first finding to biochemically connect $cPLA_2$ pathway to oxidation and inflammatory responses in microglial cells through LOX. Future studies should further examine the specific involvement of LOX isoforms in pro-inflammatory gene expression and regulation of ROS production in microglial cells.

$cPLA_2$ as a therapeutic target against neurological diseases

Since the 1990s, $cPLA_2$ has been demonstrated to be a favorable target for intervention against a wide range of neurological diseases. Using an experimental stroke model, Bonventre et al. was the first to show that $cPLA_2$ knockout mice suffered less ischemic damage and had smaller infarct volume after transient middle cerebral artery occlusion (Bonventre et al., 1997). Sanchez-Mejia et al. also demonstrated transgenic hAPP mice with $cPLA_2$ knockout to exhibit significantly less cognitive deficit compared to $cPLA_2$ intact transgenic hAPP mice, indicating a potential role of $cPLA_2$ in the pathogenesis of Alzheimer's disease (Sanchez-Mejia et al., 2008). AACOCF3, a non-selective $cPLA_2$ and $iPLA_2$ inhibitor, was discovered to be an effective pharmacological inhibitor of PLA_2. Due to its physicochemical properties, it can readily penetrate into cell membranes. In thrombin-stimulated platelets, in Ca^{2+} ionophore-stimulated human

monocytic cells, and in interleukin 1-stimulated mesangial cells, all liberation of AA is essentially blocked at a concentration of 5 to 20 μM (Gronich et al., 1994; Farooqui et al., 2006). Since the discovery and popularization of AACOCF3, more recent studies further demonstrated the administration of $cPLA_2$ pharmacologic inhibitors to offer protective effect against multiple neurological diseases. This includes the amelioration of focal ischemic damage in experimental stroke (Zhang et al., 2012), prevention of secondary tissue damage in experimental autoimmune encephalitis, an *in vivo* model for multiple sclerosis (Vana et al., 2011), as well as preservation of neuronal survival and retention of motor function in a mouse model of spinal cord injury (Huang et al., 2009; Liu et al., 2014).

While most of the studies suggested and focused on the action of $cPLA_2$ in the neurons in the event of neurodegeneration and neuronal apoptosis, less attention was given to the potential role of $cPLA_2$ in the microglial cells. While microglial activation plays an important role in limiting neuronal damage and phagocytosis of cellular debris and foreign pathogens, M1 activation of microglia cells can also promote microglia-induced neuronal damage and further propagate ongoing neuroinflammation. The results of this study had shed light on one potential mechanism of M1 microglial activation, which can potentially be targeted and controlled. Interestingly, inhibition of $cPLA_2$ by AACOCF3 was able to abrogate the morphological changes elicited by LPS and IFNγ in WT primary microglial cells. We believe that $cPLA_2$ inhibition not only prevents microglial activation, but more importantly, becomes a viable therapeutic strategy to impede neuronal cell death by limiting secondary neuronal damage. In this regard,

inhibiting the PLA_2 cascade has been considered an essential strategy for opposing microglial activation (Paris et al., 2000) and discovering new and synthetic inhibitors for PLA_2 will be an important future endeavor for understanding and treatment of neurological disorders (Ong et al., 2015).

SUMMARY/CONCLUSION

This study demonstrated a crucial role of $cPLA_2$ in the activation of microglial cells, specifically in LPS- and IFNγ-stimulated ROS and iNOS/NO production. In addition, $cPLA_2$ also controls the morphological transformations associated with microglial activation. Upon looking at the downstream pathways, results show that LPS- and IFNγ-induced activation of ROS/NO is dependent on LOX12/15 and not COX1/2, thus offering new insights into the signaling pathway during microglial activation. Further studies are needed to better understand the molecular mechanisms underlying $cPLA_2$ in microglial activation and how this may offer novel therapeutic options for the prevention and/or treatment of neuroinflammatory/neurodegenerative diseases.

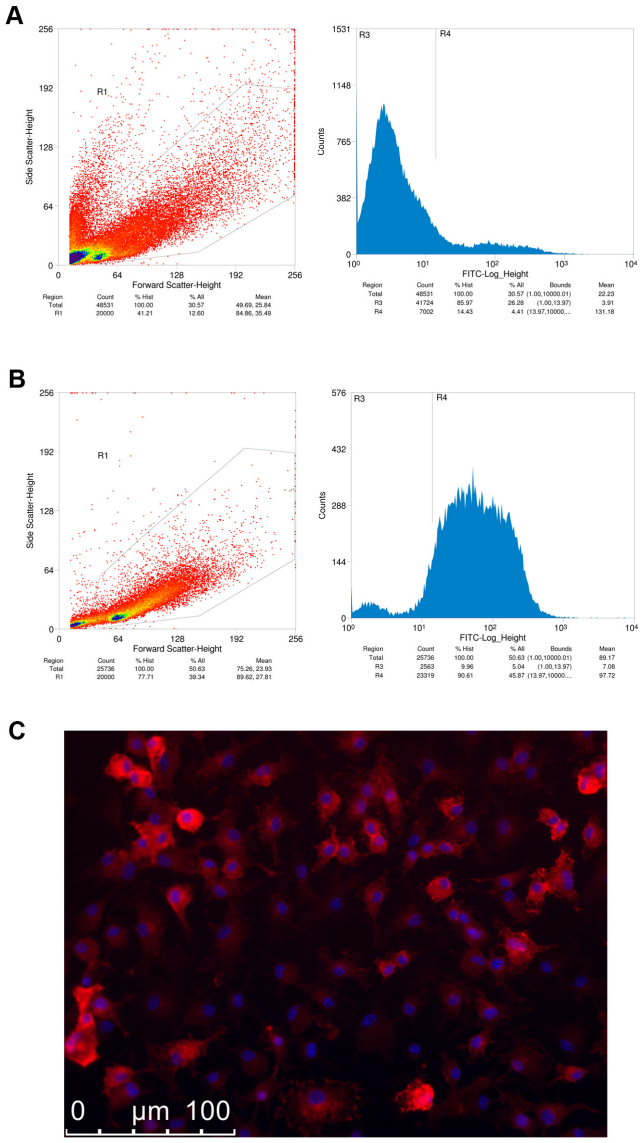

Figure 3.1

Determination of primary culture purity after isolation from brains of postnatal day 7-10 c57bl/6 mice. (A) Flow cytometry of cells from pre-sorting showed 14.43% of cells in cell suspension positive for cd11b-FITC. (B) Flow cytometry of cells from post-sorting showed 90.61% of cells in cell suspension positive for cd11b-FITC. (C) Immunocytochemical staining of cells in DIV5 primary culture showed expression of cd11b (red) in almost all cells.

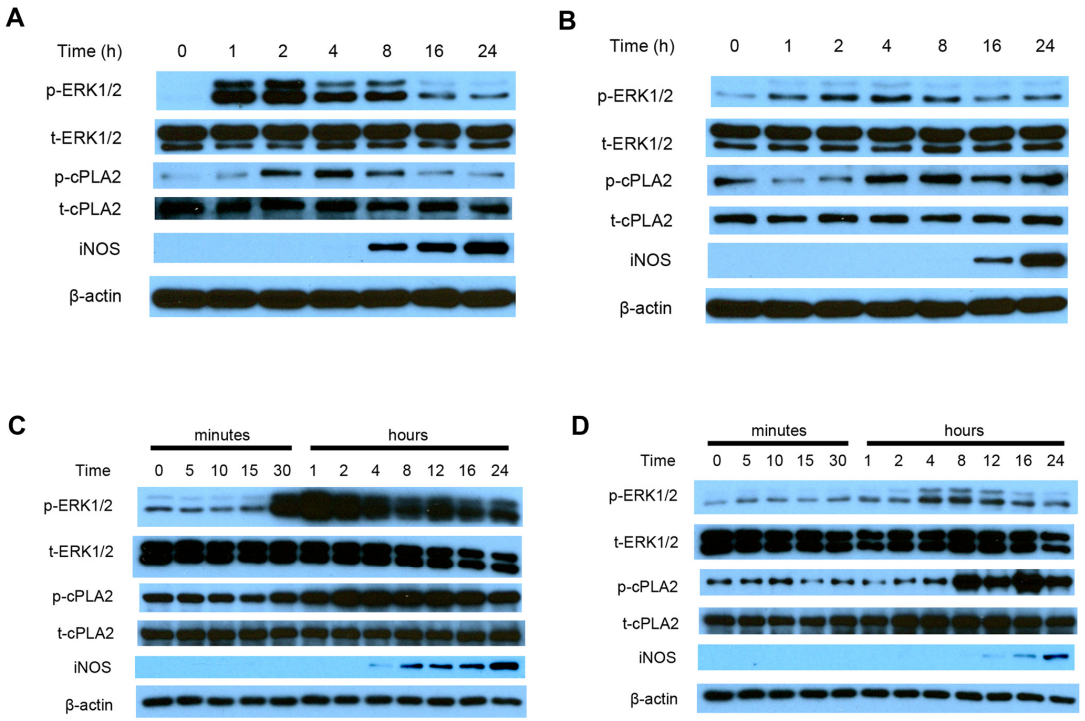

Figure 3.2

Time course of protein expression in primary microglia and BV-2 cells after LPS/IFNγ stimulation. Primary microglial cells were stimulated with (A) 200ng/mL LPS or (B) 20ng/mL IFNγ. Cells were lysed and proteins were collected and processed at the indicated time post-stimulation. Western blot was performed to determine protein expression. Similarly, the same procedure was performed with BV-2 cells stimulated with (C) 200ng/mL LPS or (D) 20ng/mL IFNγ. Results are representative blots of 2 independent time course experiments for primary microglia and 3 experiments for BV-2 cells.

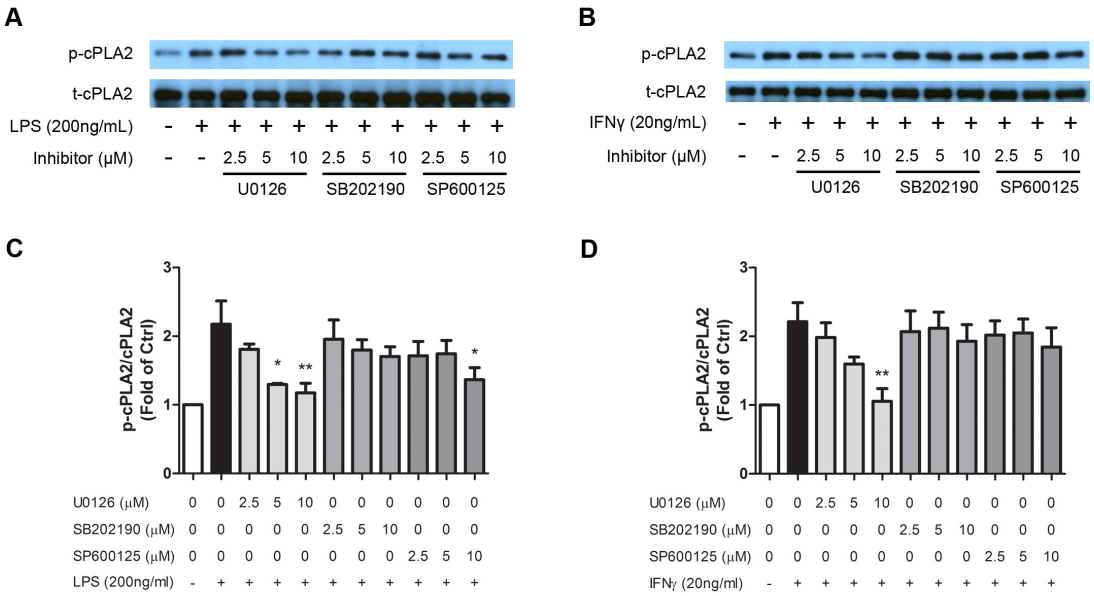

Figure 3.3

ERK1/2 contributed to cPLA$_2$ phosphorylation after LPS/IFNγ stimulation. BV-2 cells were starved for 3 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with indicated concentrations of MAPK inhibitors: U0126 (U) for ERK1/2 inhibition, SB202190 (SB) for p38 MAPK inhibition and SP600125 (SP) for JNK inhibition. Cells were then stimulated with (A, C) 200ng/mL LPS or (B, D) 20ng/mL IFNγ. Cells were lysed and proteins were collected and processed 2 hours after LPS stimulation or 8 hours after IFNγ stimulation for Western blot analyses. (A, B) Representative blots. Protein expression was quantified with QuantityOne software for 3 separate experiments for (C) LPS-stimulated BV-2 cells and (D) IFNγ-stimulated BV-2 cells. Results were expressed as the mean ± SEM (n = 3) and significant difference from the respective group was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$; **$P < 0.01$.

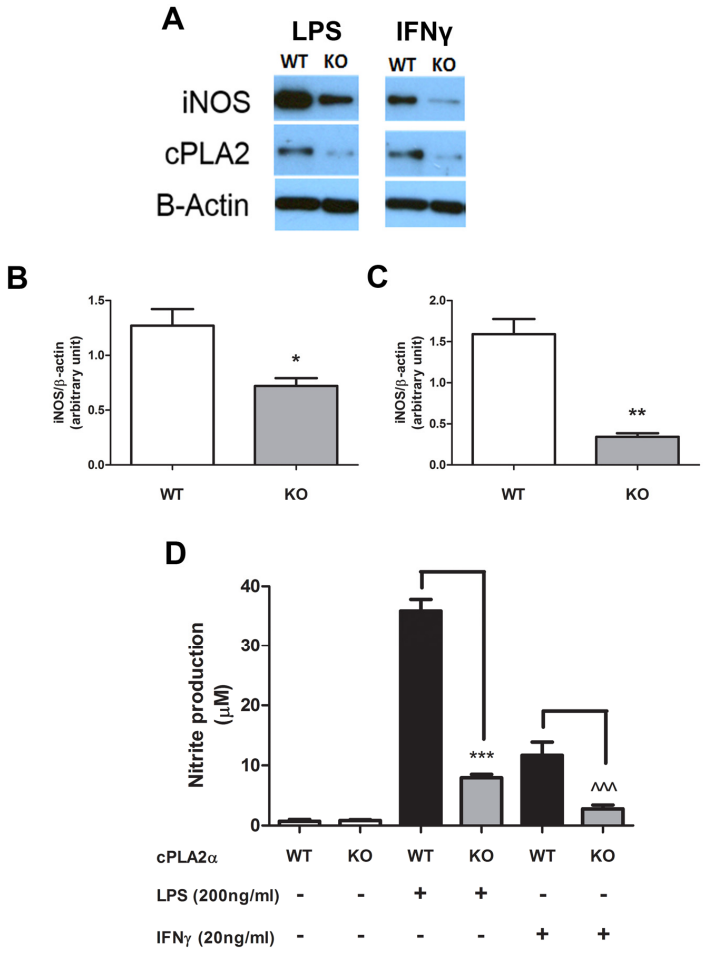

Figure 3.4

iNOS expression and NO production were significantly reduced in cPLA$_2$ KO primary microglial culture compared with WT culture. DIV5-7 primary microglial culture isolated from cPLA$_2$ KO or WT mice were stimulated with 200ng/mL LPS or 20ng/mL IFNγ for 24 hours. Cells were then lysed and proteins were collected/processed. (A) iNOS/cPLA$_2$/β-actin expressions were demonstrated by Western blot, and (B-C) iNOS/ β-actin levels were quantified with the QuantityOne software. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective paired groups was determined by t-test, * $P < 0.05$; ** $P < 0.01$. (D) Conditioned mediums from 48 hour post-stimulation samples were collected for determination of nitrite concentration with the Griess protocol. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by t-test, *** $P < 0.001$; ^^^$P < 0.001$.

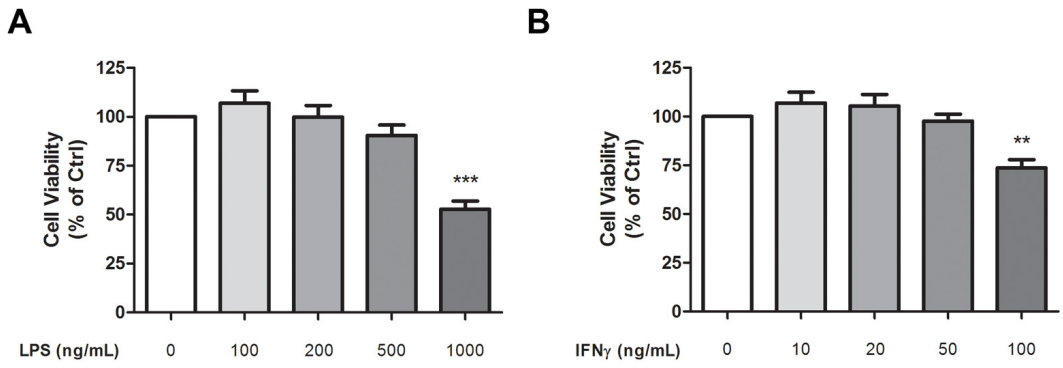

Figure 3.5

High concentrations of LPS and IFNγ were toxic to primary microglia at 24 h post-stimulation. Primary microglial cells were treated with various concentrations of either (A) LPS or (B) IFNγ. 24 h later, cell viability was measured with the WST-1 protocol as described in text. Results were expressed as the mean ± SEM (n = 3) and significant difference compared with the control group was determined by one-way ANOVA followed by Dunnett's post-tests, ** $P < 0.01$, *** $P < 0.001$.

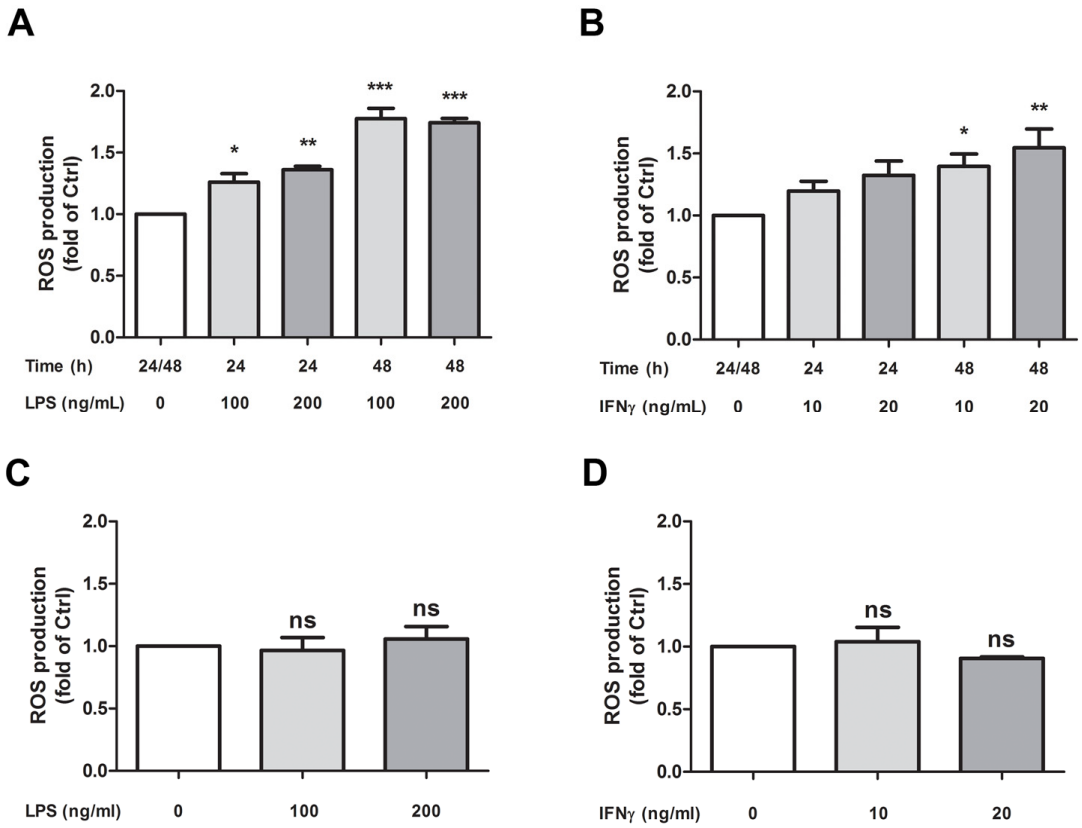

Figure 3.6

Primary microglia from cPLA$_2$ KO mice did not show a significant increase in ROS production by LPS and IFNγ. Different concentrations of (A) LPS or (B) IFNγ, as well as incubation time were used to evaluate the ROS production by primary microglia isolated from wildtype mice. Based on the results from WT culture, ROS production was evaluated at 48 hours post-stimulation with different doses of (C) LPS or (D) IFNγ in microglial cells from cPLA$_2$ KO mice. ROS production was quantified by CM-H2DCFDA fluorescence as described in the text. Results were expressed as the mean ± SEM (n = 3 WT LPS, n = 8 WT IFNγ, n = 3 KO LPS, n = 3 KO IFNγ) and significant difference between the groups was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

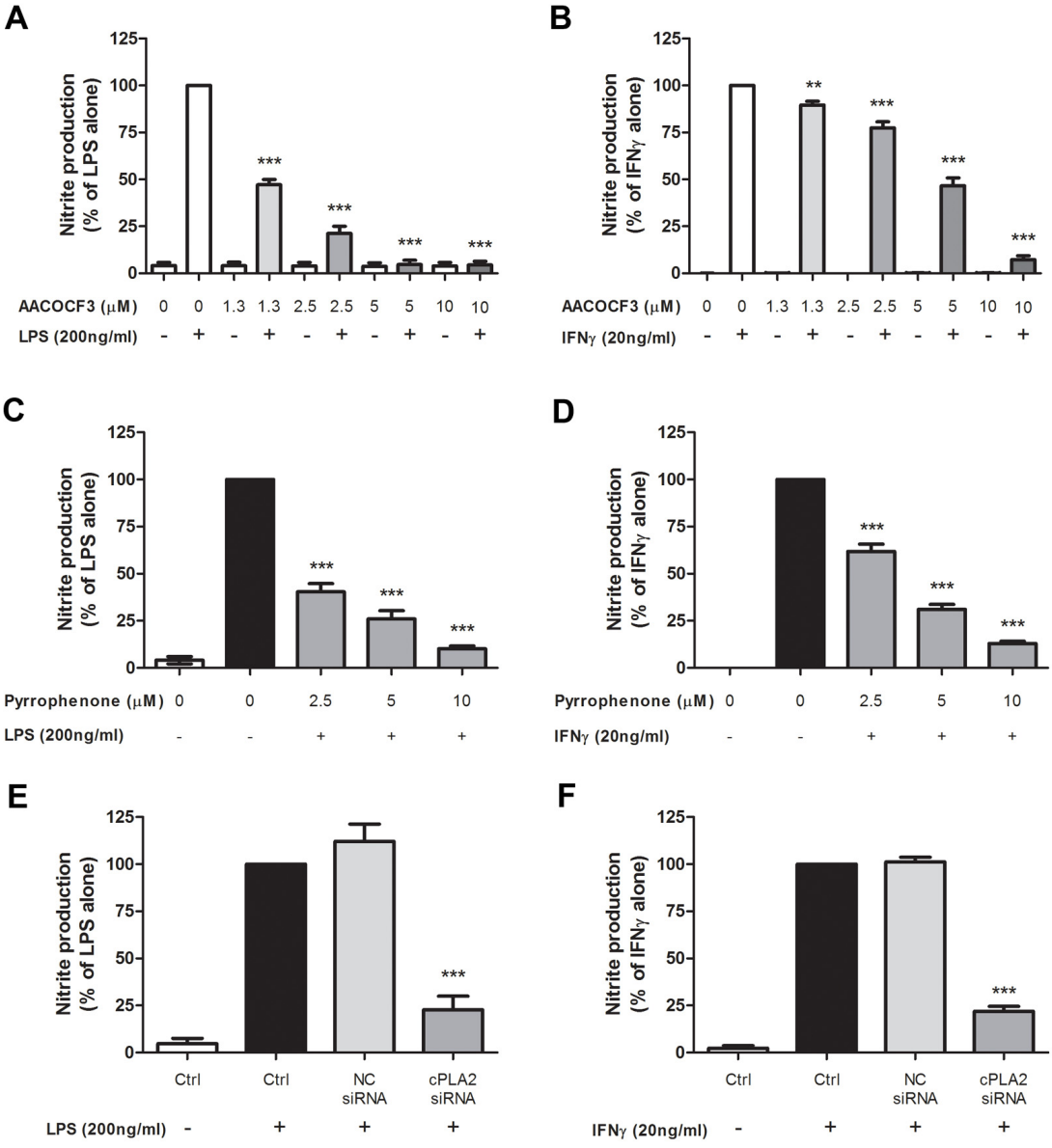

Figure 3.7

NO production in BV-2 cells after LPS or IFNγ stimulation was inhibited by cPLA$_2$ pharmacological inhibitors or siRNA knockdown. BV-2 cells were starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of cPLA$_2$ inhibitors: (A-B) AACOCF3 or (C-D) pyrrophenone.

Cells were then stimulated with (A, C) 200ng/mL LPS or (B, D) 20ng/mL IFNγ. Alternatively, BV-2 cells were transfected with siRNA against $cPLA_2$ for 24 hours before being stimulated with (E) 200ng/mL LPS or (F) 20ng/mL IFNγ. For all experiments, conditioned mediums were collected 16 hours post-stimulation and NO concentrations were measured by Griess protocol as described in the text. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, ** $P < 0.01$, *** $P < 0.001$.

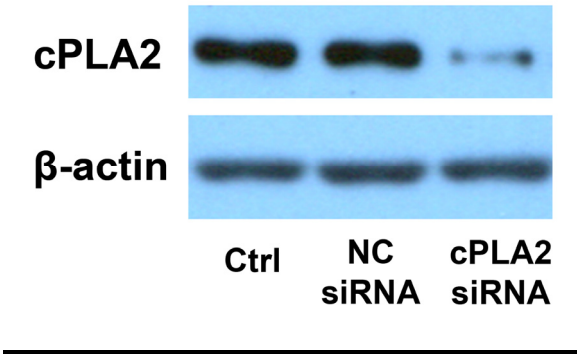

Figure 3.8

cPLA$_2$ protein expression level decreased significantly after siRNA knockdown. Representative blot demonstrating protein levels of cPLA$_2$ and β-actin in BV-2 cells between groups: (1) control, (2) BV-2 cells were transfected with negative control siRNA for 24 hours, and (3) BV-2 cells were transfected with siRNA against cPLA$_2$ for 24 hours.

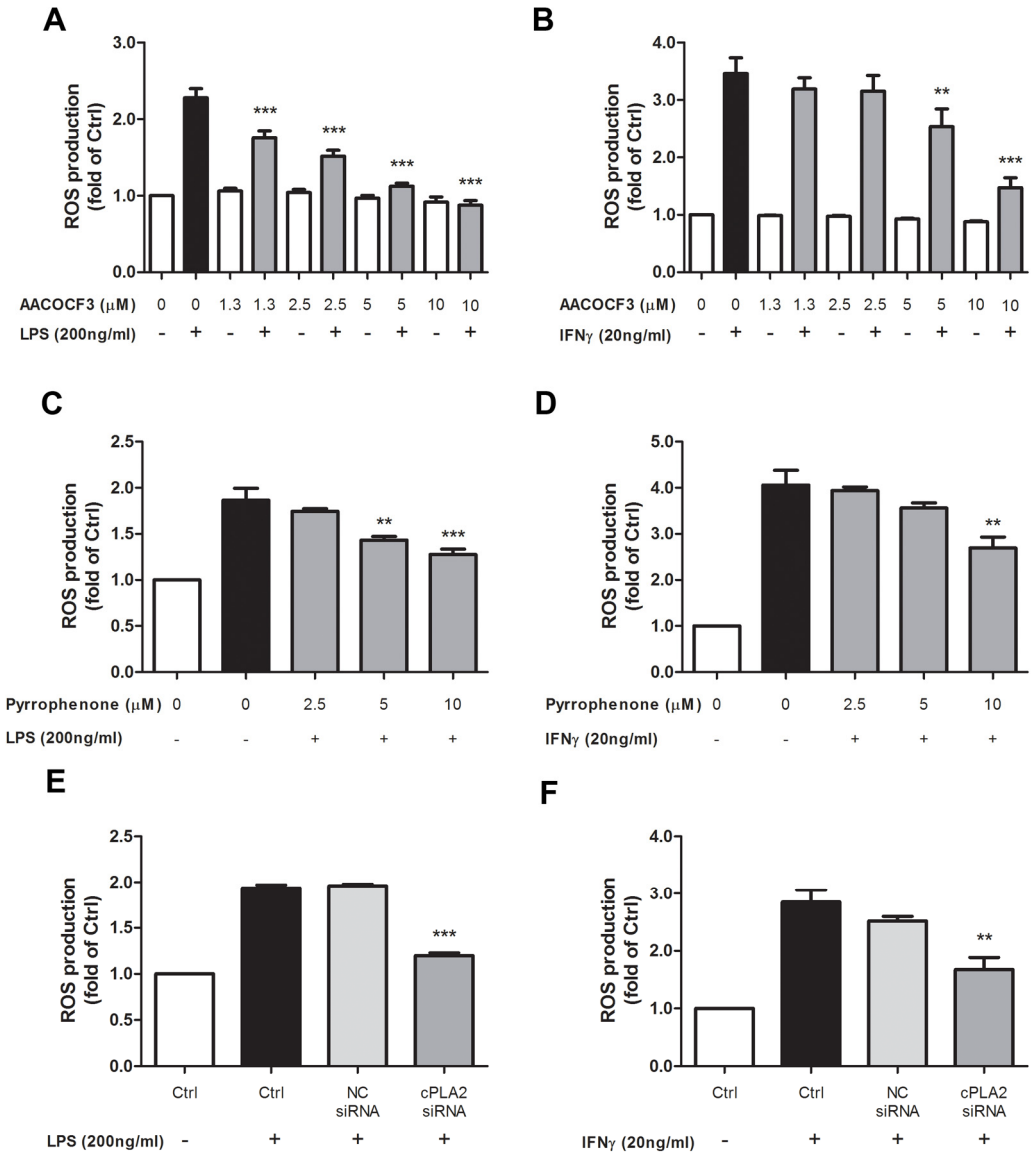

Figure 3.9

ROS production in BV-2 cells after LPS or IFNγ stimulation was inhibited by cPLA$_2$ pharmacological inhibitors or siRNA knockdown. BV-2 cells were starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of cPLA$_2$ inhibitors: (A-B) AACOCF3 or (C-D) pyrrophenone. Cells were then stimulated with (A, C) 200ng/mL LPS or (B, D) 20ng/mL

IFNγ. Alternatively, BV-2 cells were transfected with siRNA against $cPLA_2$ 24 hours before being stimulated with (E) LPS or (F) IFNγ. For all experiments, ROS production was measured 12 hours post-stimulation by CM-H2DCFDA fluorescence as described in the text. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, ** $P < 0.01$, *** $P < 0.001$.

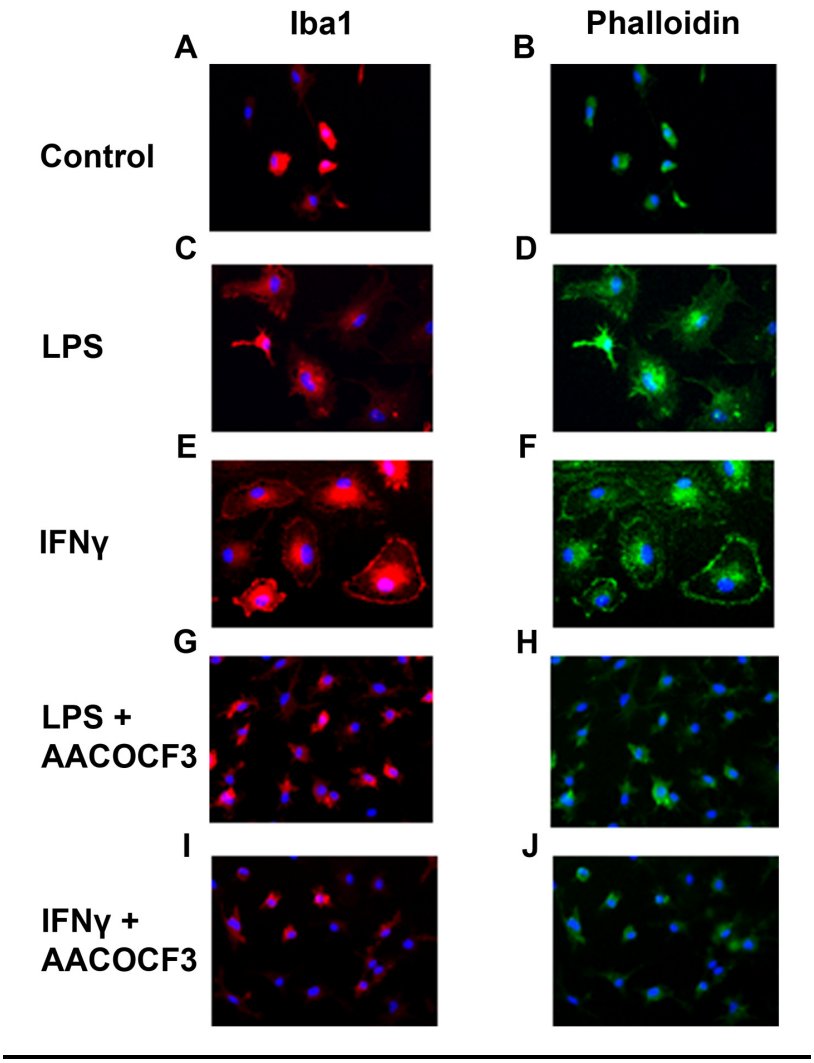

Figure 3.10

cPLA$_2$ inhibition with AACOCF3 prevented morphological transformation of primary microglia associated with M1 activation. Immunocytochemical staining for Iba-1 and phalloidin was performed with DIV5 primary microglia culture with the following treatments for 24 hours: (A, B) control, (C, D) 200ng/mL LPS, (E,F) 20ng/mL IFNγ, (G,H) 1 hour of 5μM AACOCF3 pretreatment with 200ng/mL LPS for 24 hours, (I, J) 1 hour of 5μM AACOCF3 pretreatment with 20ng/mL IFNγ for 24 hours. Iba-1

expression is represented by red fluorescent signal, phalliodin expression is represented by green fluorescent signal, and nuclear Hoechst staining is demonstrated in blue.

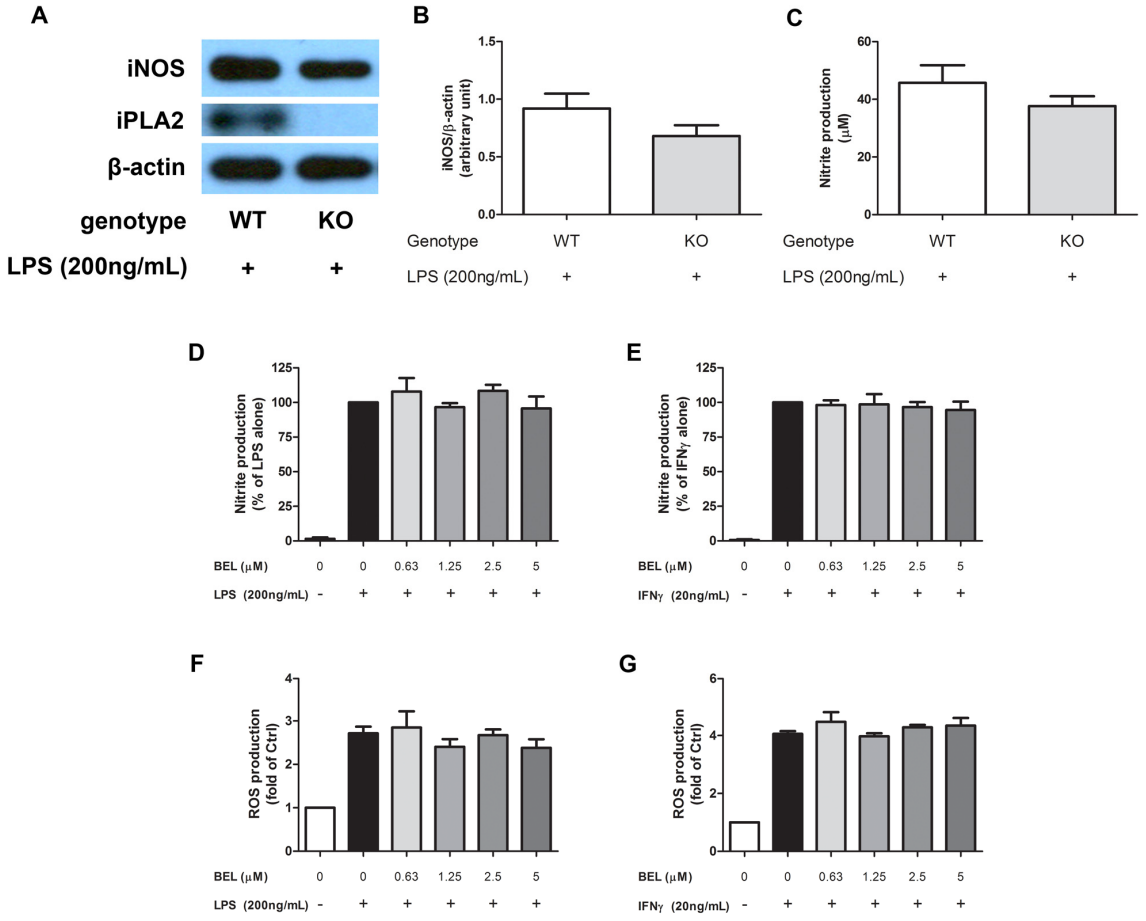

Figure 3.11

iPLA$_2$ KO primary microglial culture did not alter LPS-induced NO/ROS production. DIV5-7 primary microglial culture isolated from iPLA$_2$β KO or WT mice were stimulated with 200ng/mL LPS for 24 hours. Cells were then lysed and proteins were collected/processed. (A) iNOS/cPLA$_2$/β-actin expressions were demonstrated by Western blot, and (B) iNOS/ β-actin levels were quantified with the QuantityOne software. (C) Conditioned media from 48 hours post-stimulated samples were collected for determination of nitrite concentration with the Griess protocol. (D-G) BV-2 cells were serum-starved for 3 hour followed by incubation with indicated concentrations of BEL for 1 hour before stimulated with (D,F) 200ng/mL LPS or (E,G) IFNγ. (D-E) NO

production was measured in conditioned medium 16 hours post-stimulation by Griess protocol. (F-G) ROS production was measured 12 hours post-stimulation by CM-H2DCFDA. Results were expressed as the mean ± SEM (n = 3) and significant difference between the groups was determined by t-test (primary microglia) and one-way ANOVA followed by Dunnett's post-tests (BV-2).

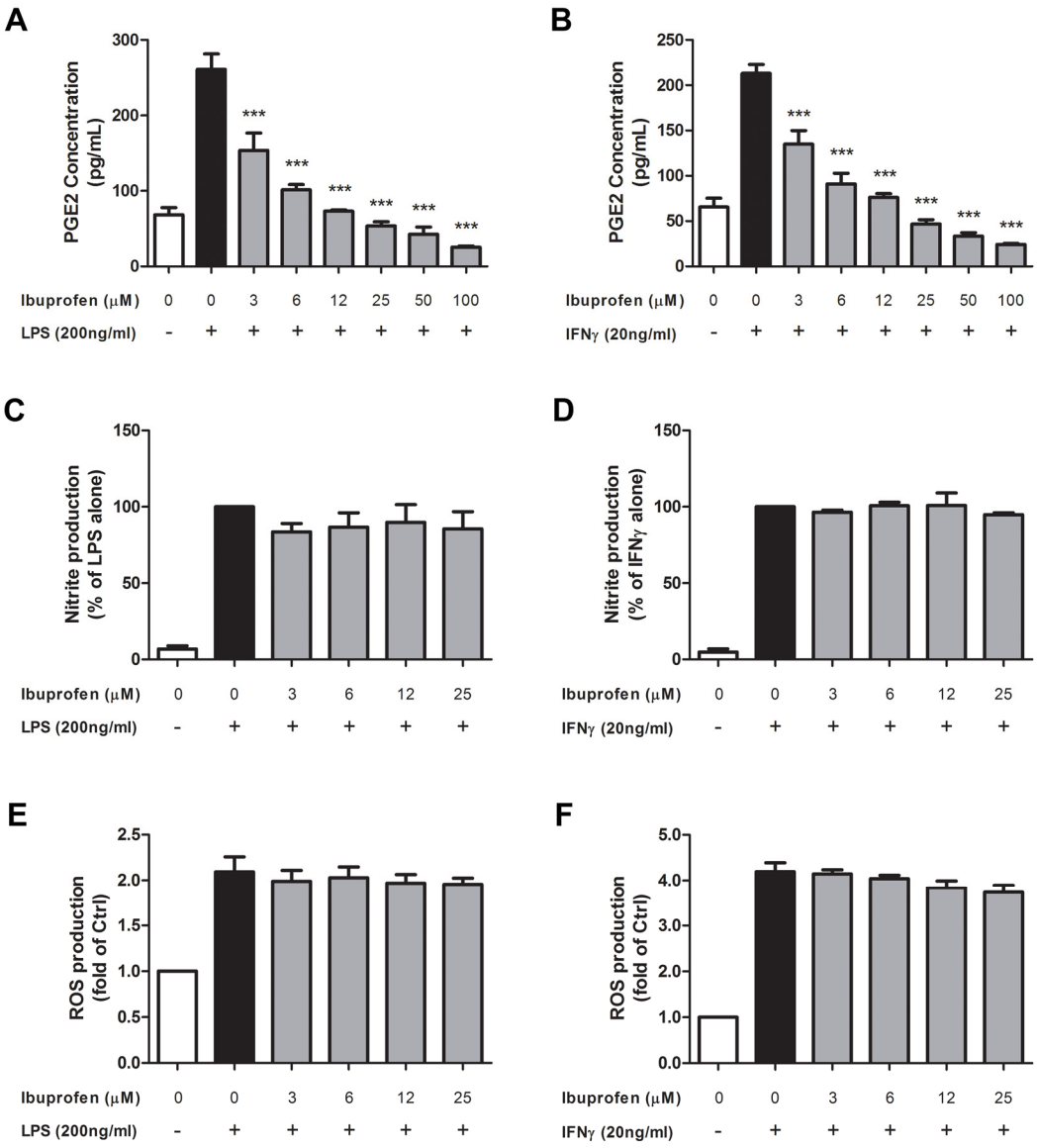

Figure 3.12

Ibuprofen dose-dependently inhibited the PGE2 production after LPS/IFNγ stimulation but did not affect the NO/ROS production in BV-2 cells. BV-2 cells were serum-starved for 3 hours followed by incubation with indicated concentrations of ibuprofen for 1 hour before stimulation with (A, C, E) 200ng/mL of LPS or (B, D, F) 20ng/mL of IFNγ. (A-B) PGE2 production was measured in conditioned medium 16

hours post-stimulation by the PGE2 ELISA assay as described in text. (C-D) NO production was measured in conditioned medium 16 hours post-stimulation by Griess protocol. (E-F) ROS production was measured 12 hours post-stimulation by CM-H2DCFDA. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, *** $P < 0.001$.

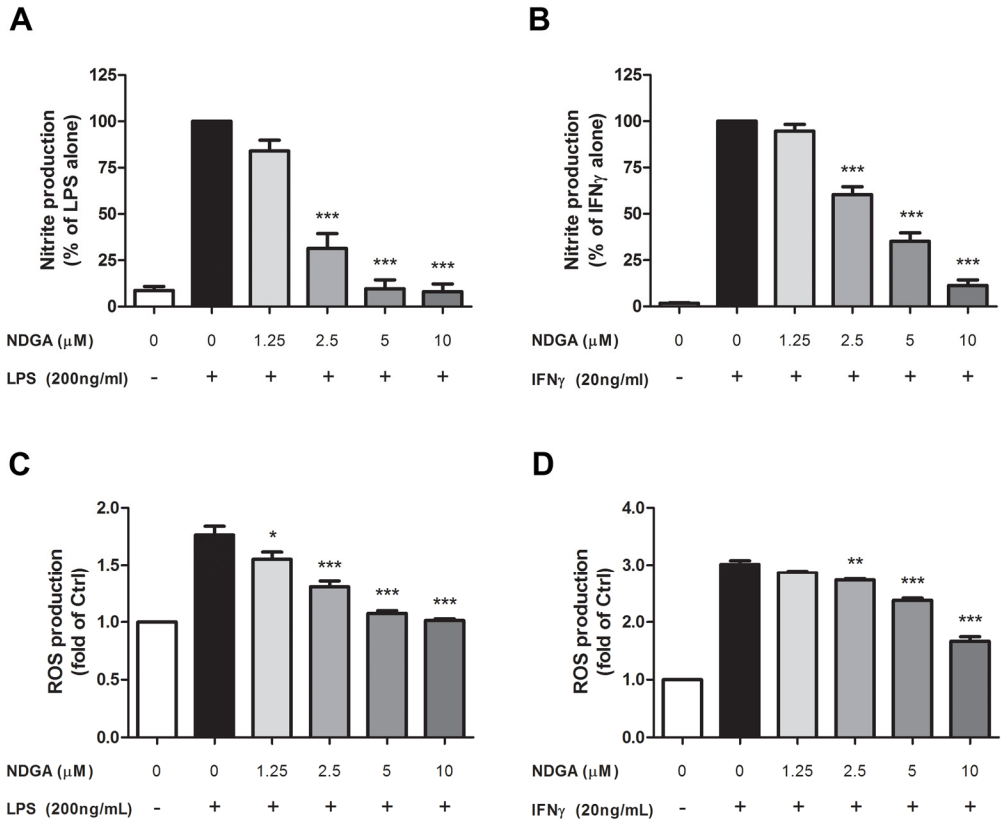

Figure 3.13

LOX inhibition mitigated NO or ROS production in BV-2 cells after LPS or IFNγ stimulation. BV-2 cells were serum-starved for 3 hour followed by incubation with indicated concentrations of NDGA for 1 hour before stimulated with (A, C) 200ng/mL LPS or (B, D) 20ng/mL IFNγ. (A-B) NO production was measured in conditioned medium 16 hours post-stimulation by Griess protocol. (C-D) ROS production was measured 12 hours post-stimulation by CM-H2DCFDA. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

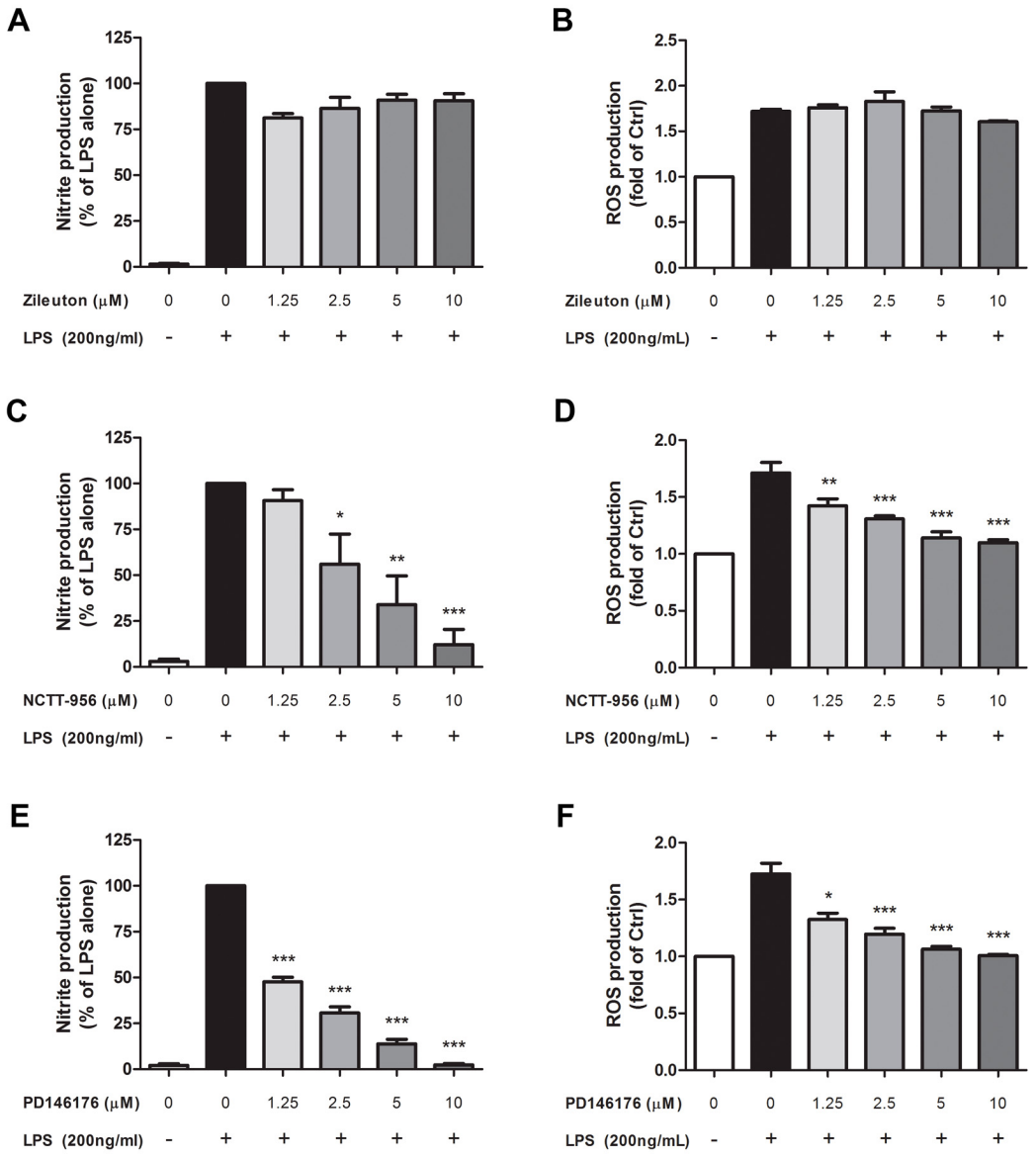

Figure 3.14

NO/ROS production by BV-2 cells after LPS stimulation was mitigated by LOX-12/15 inhibition, but not by LOX-5 inhibition. BV-2 cells were serum-starved for 3 hour followed by 1-hour incubation with indicated concentrations of LOX inhibitors: (A-B) Zileuton for LOX-5 inhibition, (C-D) NCTT-956 for LOX-12 inhibition, and (E-F) PD146176 for LOX-15 inhibition. The cells were then stimulated with 200ng/mL LPS.

(A, C, E) NO production was measured in conditioned medium 16 hours post-stimulation by Griess protocol. (B, D, F) ROS production was measured 12 hours post-stimulation with CM-H2DCFDA fluorescence. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

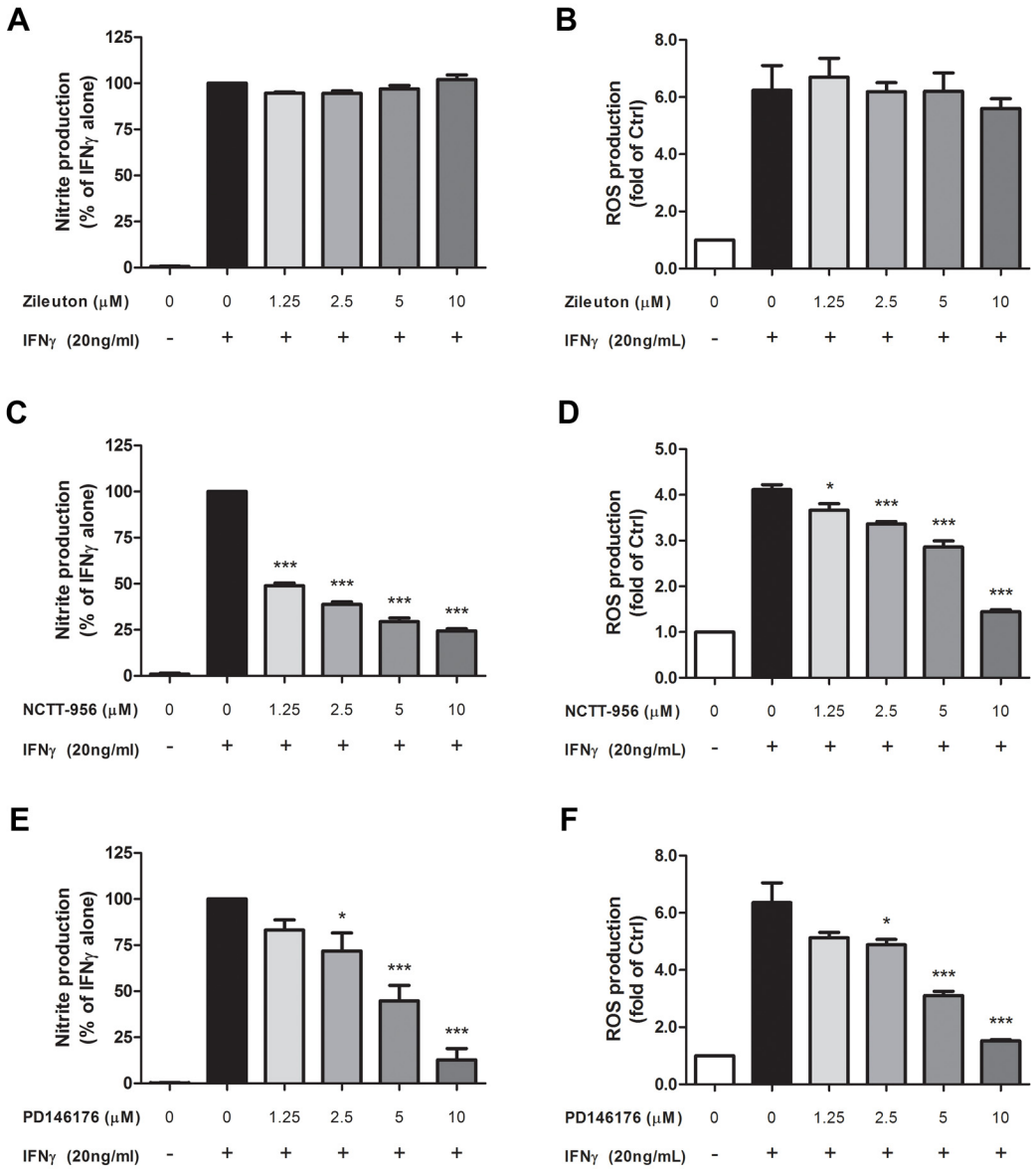

Figure 3.15

NO/ROS production by BV-2 cells after IFNγ stimulation was mitigated by LOX-12/15 inhibition, but not by LOX-5 inhibition. BV-2 cells were serum-starved for 3 hour followed by 1-hour incubation with indicated concentrations of LOX inhibitors: (A-B) Zileuton for LOX-5 inhibition, (C-D) NCTT-956 for LOX-12 inhibition, and (E-F) PD146176 for LOX-15 inhibition. The cells were then stimulated with 20ng/mL IFNγ.

(A, C, E) NO production was measured in conditioned medium 16 hours post-stimulation by Griess protocol. (B, D, F) ROS production was measured 12 hours post-stimulation with CM-H2DCFDA fluorescence. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$; *** $P < 0.001$.

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CHAPTER 4

BOTANICAL POLYPHENOLS MITIGATE MICROGLIAL ACTIVATION AND MICROGLIA-INDUCED NEUROTOXICITY: ROLE OF CYTOSOLIC PHOSPHOLIPASE A2

ABSTRACT

Microglia play a significant role in the generation and propagation of oxidative/nitrosative stress, and are the basis of neuroinflammatory responses in the central nervous system. Upon stimulation by endotoxins such as lipopolysaccharides (LPS), these cells release pro-inflammatory factors which can exert harmful effects on surrounding neurons, leading to secondary neuronal damage and cell death. Our previous studies demonstrated effects of botanical polyphenols to mitigate inflammatory responses induced by LPS and high-lighted an important role for cytosolic phospholipase A_2 ($cPLA_2$) upstream of the pro-inflammatory pathways (Chuang et al. 2015). In this study, we investigate action of botanical compounds and assess whether suppression of $cPLA_2$ in microglia is involved in the neurotoxic effects on neurons. Differentiated SH-SY5Y neuroblastoma cells were used to test neurotoxicity of conditioned medium from stimulated microglial cells, and WST-1 assay was used to assess for cell viability of SH-SY5Y cells. Botanicals such as quercetin and honokiol (but not cyanidin-3-O-glucoside, 3CG) were effective in inhibiting LPS-induced nitric oxide (NO) production and phosphorylation of $cPLA_2$. Conditioned medium from BV-2 cells stimulated with LPS or IFNɤ caused neurotoxicity to SH-SY5Y cells. Decrease in cell viability could be

ameliorated by pharmacological inhibitors for $cPLA_2$ as well as by down-regulating $cPLA_2$ with siRNA. Botanicals effective in inhibition of LPS-induced NO and $cPLA_2$ phosphorylation were also effective in ameliorating microglial-induced neurotoxicity. Results demonstrated cytotoxic factors from activated microglial cells to cause damaging effects to neurons and potential use of botanical polyphenols to ameliorate the neurotoxic effects.

INTRODUCTION

Neuroinflammation underlies the pathophysiology of numerous neurological and psychiatric conditions, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, and even schizophrenia (Glass et al. 2010; Gonzalez-Scarano and Martin-Garcia 2005). Microglia, the resident immune cells in the central nervous system, is recognized as an important cell type for propagation of inflammatory responses in the brain (Block and Hong 2005; Block et al. 2007; Aguzzi et al. 2013). Bacterial endotoxin LPS is traditionally established to activate the Toll-like receptor 2/4 and is coupled to the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway to cause transcription/translation of a number of inflammatory genes, including inducible nitric oxide synthase (iNOS). In BV-2 microglial cells, interferon-γ (IFNγ) acting through the IFNγ receptor and coupled to the canonical Janus kinase - Signal Transducer and Activator of Transcription (JAK-STAT) pathway can also induce transcription pathways for production of iNOS/nitric oxide (NO). Our study provided evidence for a cross-talk mechanism between the two pathways, with common activated components identified, such as extracellular-signal-regulated kinases 1/2 (ERK1/2), cytosolic phospholipase A_2

(cPLA$_2$) and the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (Chuang et al. 2013; Chuang et al. 2015; Sheng et al. 2011).

Many botanical compounds, especially the polyphenols from fruits and vegetables, have been shown to exert beneficial effects against neurological conditions (A. Y. Sun et al. 2011). While the mechanism of action of these botanicals may be multi-faceted, many have pointed to the anti-inflammatory and anti-oxidative properties of these compounds (Galli et al. 2002; A. Y. Sun et al. 2008). Previous studies from our laboratory as well as from others have demonstrated effects of botanicals, such as quercetin from berries and honokiol from the magnolia bark, to mitigate microglial induced production of NO and reactive oxygen species (ROS) upon stimulation by LPS or pro-inflammatory cytokines (Chuang et al. 2013; Jiang et al. 2014). These studies also unveil differences in potency in their ability to mitigate anti-oxidative and anti-inflammatory responses (Simonyi et al. 2015; G. Y. Sun et al. 2015).

The phospholipases A$_2$ (PLA$_2$) family, which is comprised mainly of cytosolic phospholipase A$_2$ (cPLA$_2$), calcium-independent phospholipase A2 (iPLA$_2$) and secretory phospholipase A2 (sPLA$_2$), has the ability to hydrolyze fatty acids in the sn-2 position of membrane phospholipids, and release polyunsaturated fatty acids, including arachidonic acid (AA) and docosahexaenoic acid (DHA) (Burke and Dennis 2009; Leslie 2015). While DHA is considered as an essential fatty acid with beneficial health effects, metabolism of AA by cyclooxygenases (COX-1 and COX-2) and lipoxygenases (LOX) to produce prostaglandins and leukotrienes is a well-established mechanism for

inflammatory responses in the biological systems (Calder 2008). Works done in our laboratory, as well as others, have suggested $cPLA_2$ to play an important role in the oxidative and nitrosative stress responses in activated microglia cells (Chuang et al. 2015; Ribeiro et al. 2013). Besides playing an integral part in the pathway involving NF-κB → ERK1/2 → $cPLA_2$ → NADPH oxidase/ROS → iNOS/NO, our study also demonstrated possible link from $cPLA_2$ to downstream components through the action of lipoxygenase-12/15 in BV-2 microglial cells (Chuang et al. 2015).

In this study, we test whether botanicals that are effective in mitigating LPS- or IFNγ-mediated inflammatory pathways also involve suppression of $cPLA_2$ phosphorylation and activation, and whether suppression of LPS-induced inflammatory responses can mitigate microglia-induced toxicity to neurons. Human neuroblastoma cells (SH-SY5Y) that are differentiated into neuron-like phenotype by retinoic acid were exposed to conditioned media from microglia treated with LPS/IFNγ as well as botanicals and $cPLA_2$ inhibitors. Our results demonstrated the role of $cPLA_2$ in microglia inflammation responses and ability for botanicals to protect neurons from toxic effects induced by activated microglial cells.

MATERIALS AND METHODS

Materials

Culture mediums include the following: Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and 0.25% (w/v) trypsin/EDTA from GIBCO

(Gaithersburg, MD), and endotoxin-free fetal bovine serum from Atlanta Biologicals (Lawrenceville, GA). LPS (rough strains) from Escherichia coli F583 (Rd mutant) was purchased from Sigma-Aldrich (St. Louis, MO). IFNγ was purchased from R & D Systems (Minneapolis, MN). Retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO). Quercetin (Sigma-Aldrich, St. Louis, MO) and cyanidin 3-O-glucoside (Indofine Chemical Comp., Hillsborough, NJ), Honokiol (lot # M8P0236) and magnolol (lot # M8F3374) (≧98% pure based on HPLC) (Nacalai Tesque, Inc, Kyoto, Japan) were dissolved in DMSO as stock solution. Pharmacological inhibitors used in this study including arachidonyl trifluoromethyl ketone (AACOCF3) and Pyrrophenone were purchased from Cayman Chemical (Ann Arbor, MI). siRNA against $cPLA_2$ Mm_Pla2g4a_8 FlexiTube siRNA (NM_008869) and AllStars Negative Control siRNA were purchased from Qiagen (Hilden, Germany). RNA interference Lipofectamine RNAiMAX Transfection Reagent was from Life Technology (Carlsbad, CA). Antibodies used for Western blots studies include: rabbit polyclonal anti-p-$cPLA_2$ (Cell Signaling, Beverly, MA), anti-$cPLA_2$ rabbit polyclonal, goat anti-mouse IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). WST-1 assay was purchased from Clontech (Mountain View, CA).

Immortalized Microglial BV-2 Cell Culture

The murine BV-2 cell line, generated by infecting primary microglia cells with a v-raf/v-myc oncogene carrying retrovirus (J2), was obtained as a gift from Dr. R. Donato (University of Perugia, Italy) and prepared as previously described (Shen et al. 2005). Briefly, BV-2 cells were cultured in DMEM (high glucose) supplemented with 10% FBS,

containing 100 units/mL penicillin and 100 µg/mL streptomycin. Cultures are maintained in 5% CO_2 incubator at 37°C. For subculture, cells were gently scraped off from the culture plates, re-suspended in pre-warmed culture medium, seeded in 6/96-well plates, and incubated until 80-90% confluence for experiments. For experiments, BV-2 cells were typically used between passages 7-12.

Human Neuroblastoma SH-SY5Y Cell Line

The immortalized human neuroblastoma cells, SH-SY5Y, were obtained from American Type Culture Collection (Manassas, VA). SH-SY5Y cells were sub-cultured by suspension in Trypsin-EDTA (0.25%) at room temperature for 1 minute to detach cells from plate. Fresh DMEM/F12 medium supplemented with 10% FBS were added to deactivate trypsin, followed by centrifugation. Medium was aspirated and cells were resuspended in 1:1 mix of DMEM/F12 supplemented with 10% FBS containing 100 units/mL penicillin and 100 µg/mL streptomycin, seeded in 96-well plates, and maintained in 5% CO_2 at 37°C. Two days after plating, culture medium was replaced with 1:1 mix of DMEM/F12 supplemented with 3% FBS, 10µM retinoic acid and penicillin/streptomycin. Retinoic acid supplemented culture medium was renewed every 3 days. Culture was allowed 5 days for differentiation prior to use. SH-SY5Y cells were used between passages 4-8 in this experiment. Cell condition and morphology were assessed by using a phase contrast Nikon DIAPHOT 300 microscope equipped with a CCD cool camera. MagnaFire2.1C software was used for microscopic image capture and processing. Representative bright field pictures were captured under a 20x objective lens.

cPLA$_2$ RNA Interference Knockdown in BV-2 Cells

BV-2 cells were transfected following the same protocol described previously (Chuang et al. 2015). Briefly, BV-2 cells were seeded in 24-well plates with antibiotics-free DMEM containing 10% FBS for 24 h. When culture reached 70-80% confluence, cells were transfected with either AllStars negative control siRNA (Qiagen), or cPLA$_2$ siRNA (NM_008869, Qiagen) (final concentration of 40 nM) using the RNAiMAX transfection reagent (Invitrogen) in mixture of Opti-MEM and DMEM mediums for 48 h prior to being used for experiments. This procedure resulted in a roughly 70% down-regulation of cPLA2 expression as described in previous study (Chuang et al. 2015).

NO Determination

NO released from BV-2 cells was measured using the Griess reagent protocol. In brief, BV-2 cells in 96-well plate were serum-starved in phenol red-free DMEM for 3 h, followed by incubation with varying concentrations of botanical compounds for 1 h. Cells were then incubated with LPS or IFNγ at 37˚C for 16 h. Aliquots of conditioned medium (50 μL) were incubated with 50 μL of the reagent A [1% (w/v) sulfanilamide in 5% phosphoric acid, Sigma-Aldrich] for 10 minutes at room temperature covered in dark. This was followed by addition of 50 μL of reagent B [0.1% (w/v) N-1-napthylethylenediamine dihydrochloride, Sigma-Aldrich] for 10 minutes at room temperature, protected from light, and absorbance at 543nm was read using a microplate reader (Biotek Synergy 4, Winooski, VT). Serial dilutions of sodium nitrite (0-100 μM) were used to generate the nitrite standard curve.

Assessment of Microglial-induced Neuronal Toxicity

BV-2 cells were plated in 24-well plates until confluence. Cells were serum-starved for 3 hours, pretreated with or without botanicals for 1 hour, and then stimulated with LPS or IFNγ for 24 hours. The conditioned media were collected and centrifuged at 1500rpm for 3 mins to remove cell debris. Medium of differentiated SH-SY5Y culture in 96-well plates was then replace with 100μL of cell-free conditioned medium from treated BV-2 cells, and incubated for 48 hours. Cell viability for SH-SY5Y cells was determined using the WST1 assay protocol (Clontech). Briefly, 10 μL of the WST-1 reagent was added in each well. Cells were incubated for 1 h at 37°C and absorbance was read at 450 nm (with reference wavelength at 650 nm) with a microplate reader.

Western Blot Analysis

Cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS. The cell lysate was centrifuged at 10,000 × g for 15 min at 4°C and transferred to a clean tube to remove cell debris. After measuring protein concentration in each sample using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL), 10 μg of each sample was loaded in SDS-PAGE for electrophoresis. Proteins were then transferred from gel to 0.45 μm nitrocellulose membranes. Membranes were incubated in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk for 1 h at room temperature. The membranes were then incubated at 4°C overnight with antibodies against $cPLA_2$ (1:1000) or phospho-$cPLA_2$ (1:1000). After repeated washing with 1X TBS-T, blots were incubated with goat anti-rabbit IgG-HRP (1:4000) for 1 h at room temperature. Immuno-labeling was detected by

chemiluminescence WestPico/Femto and developed in X-ray film developer. Films were scanned and the optical density of protein bands was quantified with the QuantityOne software (BioRad, Hercules, CA).

Statistical Analysis

Data were presented as means ± SEM. Results were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests (V4.00; GraphPad Prism Software Inc., San Diego, CA). Statistical significance was considered for $p < 0.05$.

RESULTS

Quercetin and Honokiol, but not Cyanindin-3-O-glucoside, Significantly Suppress LPS-induced NO Production in BV-2 Microglial Cells

Our previous studies have demonstrated the ability for LPS and IFNγ to activate NF-κB and JAK-STAT pathways and induce oxidative and nitrosative products in BV-2 microglial cells (Sheng et al. 2011). In order to assess the anti-inflammatory properties of the botanical compounds of interest, we pretreat the BV-2 cells with quercetin, honokiol, or C3G (chemical structures shown in Fig. 4.1) one hour before LPS stimulation. C3G was used as a negative control because our previous study showed that this polyphenol derivative was not effective in inhibiting LPS-induced NO in BV-2 cells (Simonyi et al. 2015). At 16 hours after LPS stimulation, we observed significant dose-dependent decrease in NO production after pretreatment with quercetin and honokiol, but not with C3G (Fig. 4.2A-C).

Quercetin and Honokiol, but not C3G, Suppress cPLA$_2$ Phosphorylation in BV-2 Cells after LPS Stimulation

In our recent study, we demonstrated cPLA$_2$ to play a significant role in the oxidative/nitrosative response in microglial cells and regulation of cPLA$_2$ phosphorylation by ERK1/2 (Chuang et al. 2015). In addition, we demonstrated that cPLA$_2$ activation is important in regulating downstream ROS production by NADPH oxidase as well as the induction of iNOS and NO (Chuang et al. 2015). In this study, we tested whether botanicals that are active in inhibiting LPS-induced NO also suppress cPLA$_2$ phosphorylation. After pretreating the BV-2 cells with the botanical compounds for 1 hour followed by stimulation with LPS, cells were lysed 4 hours post-stimulation and protein analyzed for levels of total and phospho-cPLA$_2$. As shown in Fig. 4.3, p-cPLA$_2$ level was increased after LPS stimulation, and was suppressed dose-dependently by quercetin (Fig. 4.3A-B) and honokiol (Fig. 4.3C-D). Consistent with previous results showing inability for C3G to inhibit LPS-induced NO production, this compound also did not inhibit LPS-induced phosphorylation of cPLA$_2$ (Fig. 4.3E-F).

Effects of LPS/IFNγ-stimulated Microglial Cells on Neurotoxicity in Differentiated SH-SY5Y Cells

Microglia-induced neurotoxicity is a well-established phenomenon seen in acute inflammation in the CNS (Block et al. 2007). Our recent study demonstrated pharmacologic and genetic ablation of cPLA$_2$ not only mitigated LPS/IFNγ-induced oxidative/nitrosative responses but also protected microglial cells to express the

inflammatory phenotype (Chuang et al. 2015). In this study, an *in vitro* system was used to assess whether conditioned media from activated microglial cells may offer deleterious effects on differentiated SH-SY5Y cells. Neuroblastoma SH-SY5Y cells were treated with 10 μM retinoic acid medium for 5 days. Under observation by light microscopy, undifferentiated SH-SY5Y cells appeared to be clumped with short neurites (Fig. 4.4A), whereas differentiated SH-SY5Y cells showed neuron-like morphology with neurites and dendrite formation (Fig. 4.4B). When the differentiated SH-SY5Y cells were treated with conditioned medium from BV-2 cells that were stimulated with LPS or IFNγ (24h), significant toxicity was observed after 48h of incubation (Fig. 4.4C). To verify that the microglial-mediated toxicity was due to released substances in the microglia cultured medium and not the effects of LPS or IFNγ in the conditioned medium alone, cell-free medium containing only LPS or IFNγ added to SH-SY5Y culture demonstrated no toxicity (Fig. 4.4C).

Inhibition of $cPLA_2$ Activity/expression in BV-2 Cells Resulted in a Decrease in Microglia-induced Nurotoxicity in SH-SY5Y Cells

Using the above-mentioned protocol, SH-SY5Y cells that were incubated with conditioned medium from LPS/IFNγ-stimulated BV-2 cells pretreated with $cPLA_2$ inhibitors (AACOCF3 and pyrrophenone) showed a significant decrease in cell viability (Fig. 4.5). The protective effect with AACOCF3 (Fig. 4.5A-B) appeared to be similar to the specific cPLA2 inhibitor, pyrrophenone (Fig. 4.5C-D). Similarly, SH-SY5Y cell toxicity was also reduced when SH-SY5Y cells were treated with conditioned medium prepared from stimulated BV-2 cells with $cPLA_2$ knocked down by siRNA (Fig. 4.6A-B).

Pretreatment of BV-2 Cells with Quercetin and Honokiol, but not C3G, Reduced Neurotoxicity in Differentiated SH-SY5Y Cells

As demonstrated above, botanicals such as quercetin and honokiol and not C3G inhibited LPS-induced NO production and $cPLA_2$ phosphorylation, it is reasonable to test whether these botanicals can also reduce microglial-induced neurotoxicity in SH-SY5Y cells. As shown in Fig. 4.7, conditioned media from microglia treated with quercetin or honokiol, but not C3G, were able to reduce LPS-induced toxicity in SH-SY5Y cells (Fig. 4.7). Results also showed a better protective effects with quercetin as compared with honokiol.

DISCUSSION

Studies from our laboratory as well as from others have demonstrated different botanical polyphenols (including honokiol and quercetin) to mitigate LPS and IFNγ-stimulated inflammatory responses in microglial cells (Chuang et al. 2013; Simonyi et al. 2015; G. Y. Sun et al. 2015). These studies also unveiled the involvement of Mitogen-activated protein kinases (MAPKs), e.g., ERK1/2, in regulating cross-talk between LPS-stimulated NF-κB and IFNγ-stimulated JAK-STAT transcription pathways in mediating the inflammatory responses (Chuang et al. 2013; Jiang et al. 2014). Along this line, ERK1/2 activation was also linked to phosphorylation and activation of $cPLA_2$, an important enzyme for regulating membrane phospholipids and release of AA for mediating the synthesis of eicosanoids and leukotrienoids (Chuang et al. 2015).

Interestingly, despite of polyphenols having similar properties, not all showed the same responses (Simonyi et al. 2015; G. Y. Sun et al. 2015). For example, quercetin can inhibit LPS-induced NO more potently than cyanidin in BV-2 microglial cells (G. Y. Sun et al. 2015). Furthermore, most polyphenols with glycosylated linkages were not effective in suppressing the inflammatory responses (Simonyi et al. 2015).

In this study, results showed the ability for quercetin and honokiol, but not C3G, to mitigate LPS-induced $cPLA_2$ phosphorylation in microglial cells. These results confirm our earlier study demonstrating the role of $cPLA_2$ in mediating microglial oxidative/nitrosative responses (Chuang et al. 2015). Since Toll-like receptors are known to mediate the induction of ERK-$cPLA_2$-NF-κB pathway, effects of botanicals possibly target the receptors, ERK1/2 or other targets upstream of $cPLA_2$ in this oxidative/nitrosative pathway in microglia.

SH-SY5Y is a neuroblastoma cell line originally cloned from SK-N-SH cell line derived from the bone marrow biopsy of a 4-year-old patient. In contrast to the S-type SH-EP subcloned cell line from the SK-N-SH cells, SH-SY5Y cells is N-type (neuronal), and possess the ability to form neurites, as well as the capability to differentiate into cells along the neuronal lineage (La Quaglia and Manchester 1996). Despite that SH-SY5Y cells typically grow in clusters due to its cancerous nature, treatment of retinoic acid can cause differentiation and dendrite formation, as well as limitation of unopposed cell growth. The differentiated SH-SY5Y cells exhibit a number of neuronal properties with active neurotransmitter synthetizing enzymes reflecting the dopaminergic phenotype (La

Quaglia and Manchester 1996). Therefore, these cells are well suited as a model to investigate neuron-glial cell interaction (Biedler et al. 1978). In a study by Yang et al. (2015), immortalized rat astrocytes (DITNC) stimulated with tumor necrosis factor α (TNFα) and IL-1β released $sPLA_2$-IIA (an inflammatory enzyme) into the culture medium. When the cytokine-conditioned media containing $sPLA_2$-IIA was applied to SH-SY5Y cells, there was alteration in membrane fluidity and increased secretion of amyloid precursor protein (APP) products (Yang et al. 2015). However, since murine microglial cells lack the ability to induce $sPLA_2$-IIA, this enzyme cannot be included as the cause of neurotoxicity in this experimental paradigm.

In this study, we demonstrated that neuronal toxicity in differentiated SH-SY5Y cells occurred as a result of exposure to cytotoxic products from microglia activation, as incubation with LPS or IFNγ alone did not affect neuronal survival. This is in agreement with previous studies demonstrating that activated microglia induced cytotoxic effects to SH-SY5Y neurons (Pan et al. 2008; S. Wang et al. 2011). Although specific microglial products were not identified in this study, others have suggested that neurotoxic factors, such as superoxide, myeloperoxidase, prostaglandins, NO, glutamate, IL-1β and TNFα, to play a role in causing toxicity to neurons under *in vitro* and *in vivo* models (Lull and Block 2010; B. Liu et al. 2003; Colton and Gilbert 1987; B. Liu et al. 2002; S. C. Lee et al. 1993; T. Wang et al. 2005). Our studies underline the important role of $cPLA_2$ in mediating the neuronal cytotoxic effects. Activation of $cPLA_2$ not only is important for synthesis of eicosanoids and leukotrienoids by COXs and LOXs, regulation of its phosphorylation by ERK1/2 further suggests a role of this enzyme upstream of the NF-

κB pathway for synthesis of inflammatory products (Chuang et al. 2015). In our previous study, cPLA$_2$ inhibitor AACOCF3 was shown to protect BV-2 cells form LPS- and IFNγ-induced morphological changes (Chuang et al. 2015). Therefore, it is reasonable to consider that cPLA$_2$ inhibition plays an important role in preventing neurotoxicity to SH-SY5Y cells by activated microglia. These results are in agreement with other studies demonstrating the protective effects of AACOCF3 in animal models of neurologic diseases, including experimental stroke (Zhang et al. 2012), experimental autoimmune encephalitis for multiple sclerosis (Vana et al. 2011), as well as spinal cord injury (Huang et al. 2009; N. K. Liu et al. 2014).

In this study, we selected two botanical polyphenols, honokiol and quercetin, known for their anti-oxidative and anti-inflammatory properties. Consistent with the results with cPLA$_2$ inhibitors, quercetin and honokiol not only are effective in inhibition of oxidative and nitrosative products, but can also suppress cPLA$_2$-mediate pathways for production of eicosanoids and leukotrienoids. Honokiol is a polyphenolic compound extracted from the bark of *Magnolia officinalis*, a common herbal plant in traditional Chinese and Korean medicine (Y. J. Lee et al. 2011). It has been shown to exert beneficial effects against neurological disorders, including Alzheimer's disease, Parkinson's disease, stroke, depression, and anxiety (Watanabe et al. 1983; Maruyama et al. 1998; B. Liu et al. 2005; H. H. Chen et al. 2011; Xu et al. 2008; Chang-Mu et al. 2010). Previous studies performed in our lab, as well as by others, had demonstrated anti-inflammatory effects of these compounds to mitigate production of ROS, iNOS/NO, and prostaglandins/leukotrienes in BV-2 microglial cells stimulated with LPS/IFNγ (Chuang

et al. 2015; Kuo et al. 2010; Oh et al. 2009; Wu et al. 2011). The mechanism of action was suggested to involve inhibition of ERK1/2 phosphorylation, NADPH oxidase activation/translocation, and NF-κB activation. Indeed, our recent studies demonstrated the important role of ERK1/2 in mediating a number of signaling pathways, including cross-talk between the NF-κB and JAK-STAT pathways and activation of $cPLA_2$ in microglial cells (Chuang et al. 2015; Jiang et al. 2014). Quercetin is abundant in a variety of fruits, such as berries, apple, and even onion, and its ability to inhibit LPS-induced NF-κB inflammatory pathway and NO production in microglial cells has been well recognized (J. C. Chen et al. 2005; Kang et al. 2013; Kao et al. 2010). More recent studies provided evidence that quercetin not only is effective in suppressing LPS-induced inflammatory responses, but also capable of stimulating anti-oxidant pathway and synthesis of phase II enzymes and proteins (G. Y. Sun et al. 2015; Kang et al. 2013). Studies in vivo indicated ability for quercetin to ameliorate damage against neurodegenerative diseases including cerebral ischemia (Annapurna et al. 2013; Hwang et al. 2009). These pleotropic properties of quercetin qualify it to become an adaptogen with potential as a nutraceutical for intervention in neurodegenerative disorders (Calabrese et al. 2010; J. Lee et al. 2014).

SUMMARY/CONCLUSION

This study demonstrated activation of $cPLA_2$ and increased oxidative and nitrosative products contribute to neurotoxic effects of microglial cells on SH-SY5Y neurons. Botanicals that are active in suppressing LPS-induced oxidative and

inflammatory responses in microglial cells are also effective in protecting the cytotoxic effects on SH-SY5Y neurons.

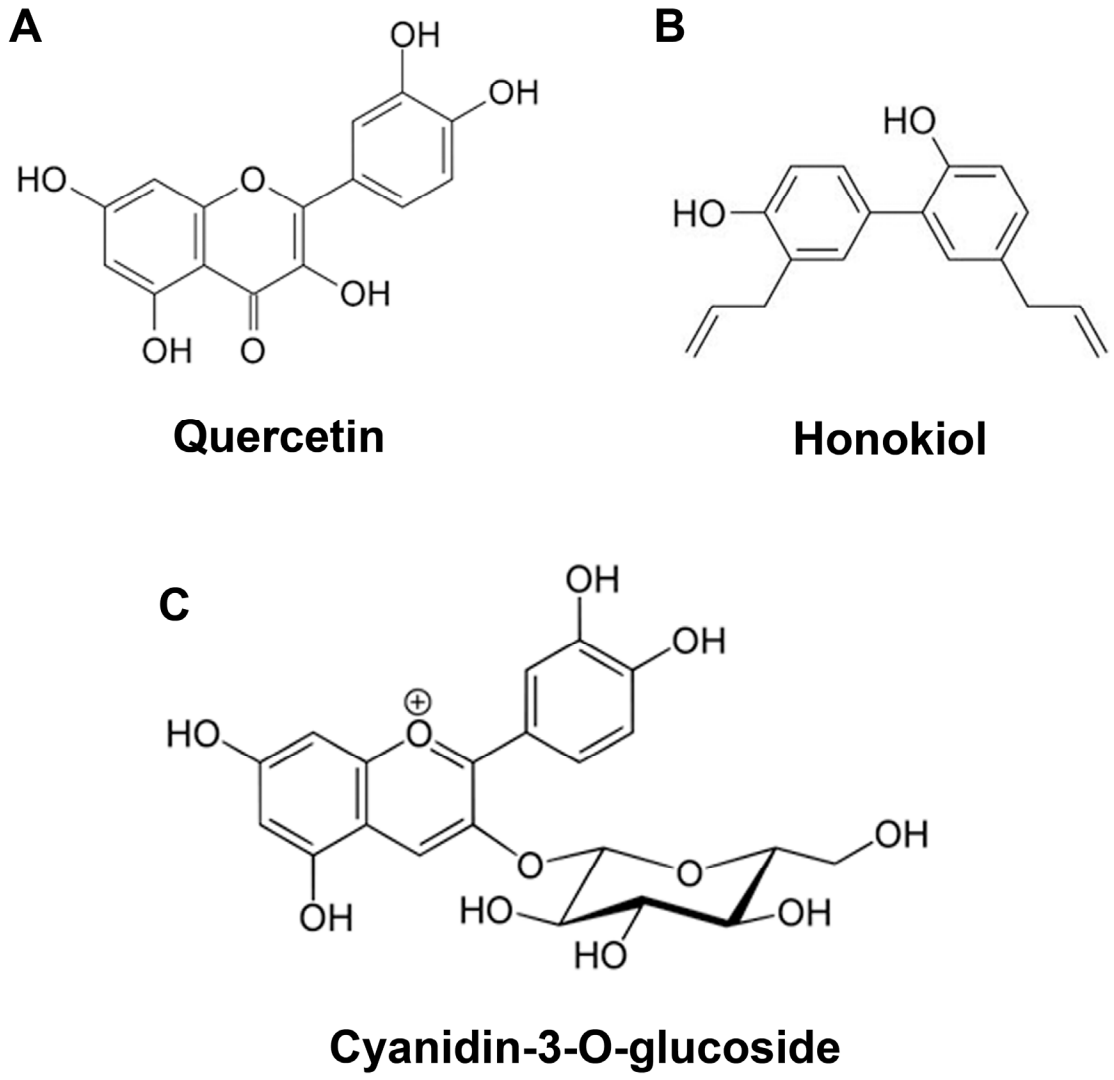

Figure 4.1

Chemical structure of (A) quercetin, (B) honokiol and (C) cyanidin-3-O-glucoside (C3G).

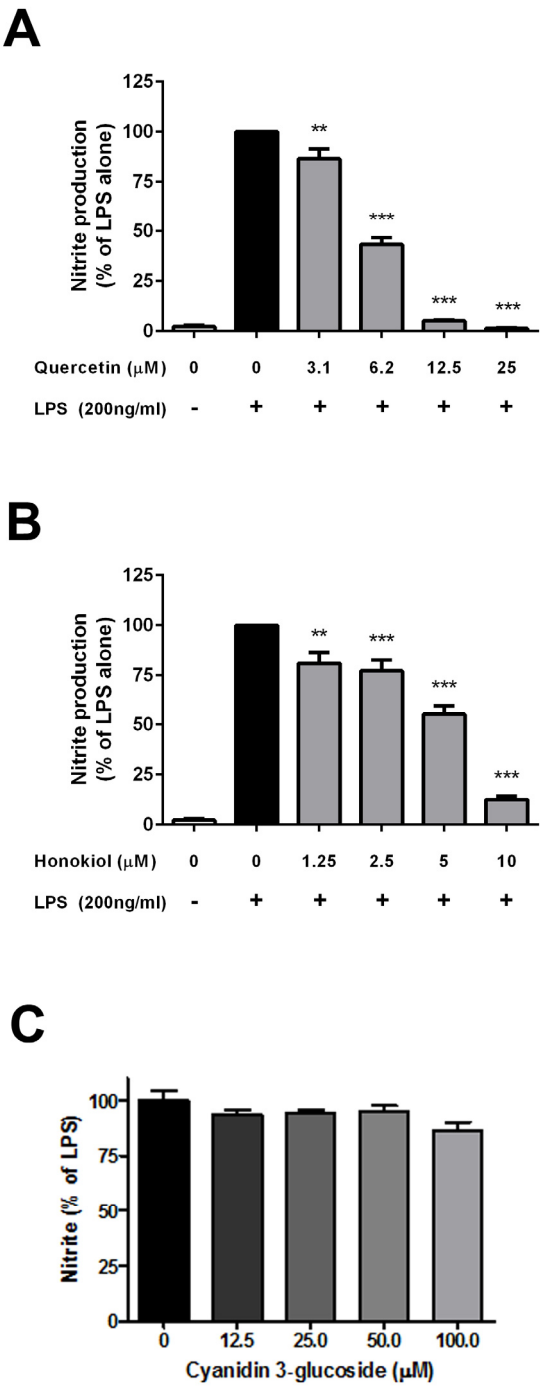

Figure 4.2

LPS-induced NO production in BV-2 cells was inhibited by quercetin, honokiol, but not C3G. BV-2 cells were serum-starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of (A) quercetin (B) honokiol, or (C) C3G (data taken from Simonyi et al., 2015). Cells were then stimulated with (A-B) 200ng/mL or (C) 100ng/mL of LPS. Conditioned media were collected 16 hours post-stimulation and NO concentrations were measured by Griess protocol as described in the text. Results were expressed as the mean ± SEM (n = 3) and significant difference between LPS and LPS + polyphenol groups was determined by one-way ANOVA followed by Dunnett's post-tests, ** $P < 0.01$, *** $P < 0.001$.

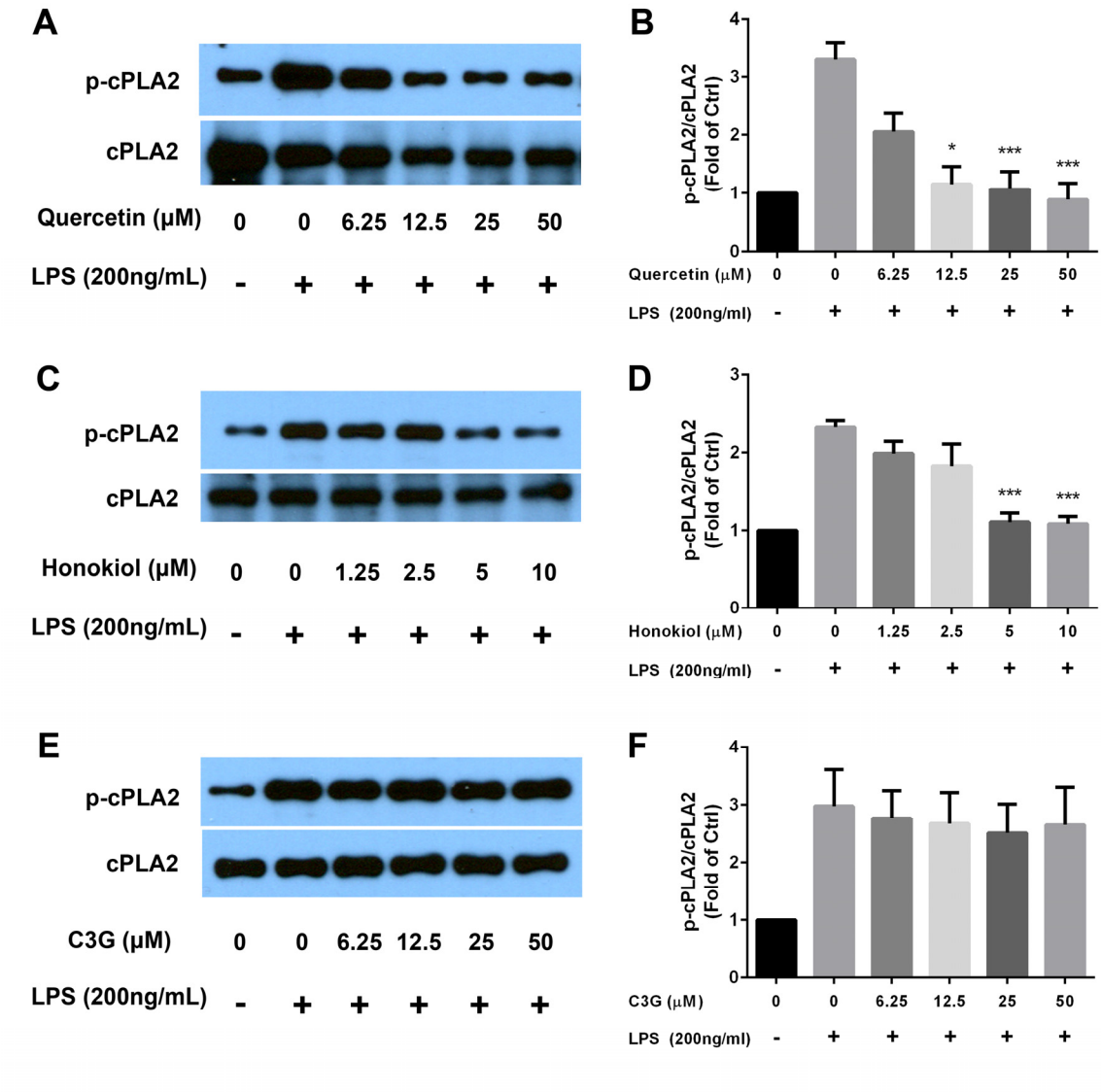

Figure 4.3

LPS-stimulated cPLA$_2$ phosphorylation in BV-2 cells was inhibited by quercetin, honokiol, but not C3G. BV-2 cells were serum-starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of (A-B) quercetin (C-D) honokiol, or (E-F) C3G. Cells were then stimulated with 200ng/mL LPS. Cells were lysed and proteins were collected and processed at 4 hours post-stimulation. Western blot was performed to determine protein

expression. cPLA2 protein band appeared just below the 100 kDa molecular weight standard. Representative blots (A, C, E) from three independent experiments, and (B, D, F), quantification of protein expression. Results were expressed as the mean ± SEM (n = 3) and significant difference between LPS and LPS + polyphenol groups was determined by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$, *** $P < 0.001$.

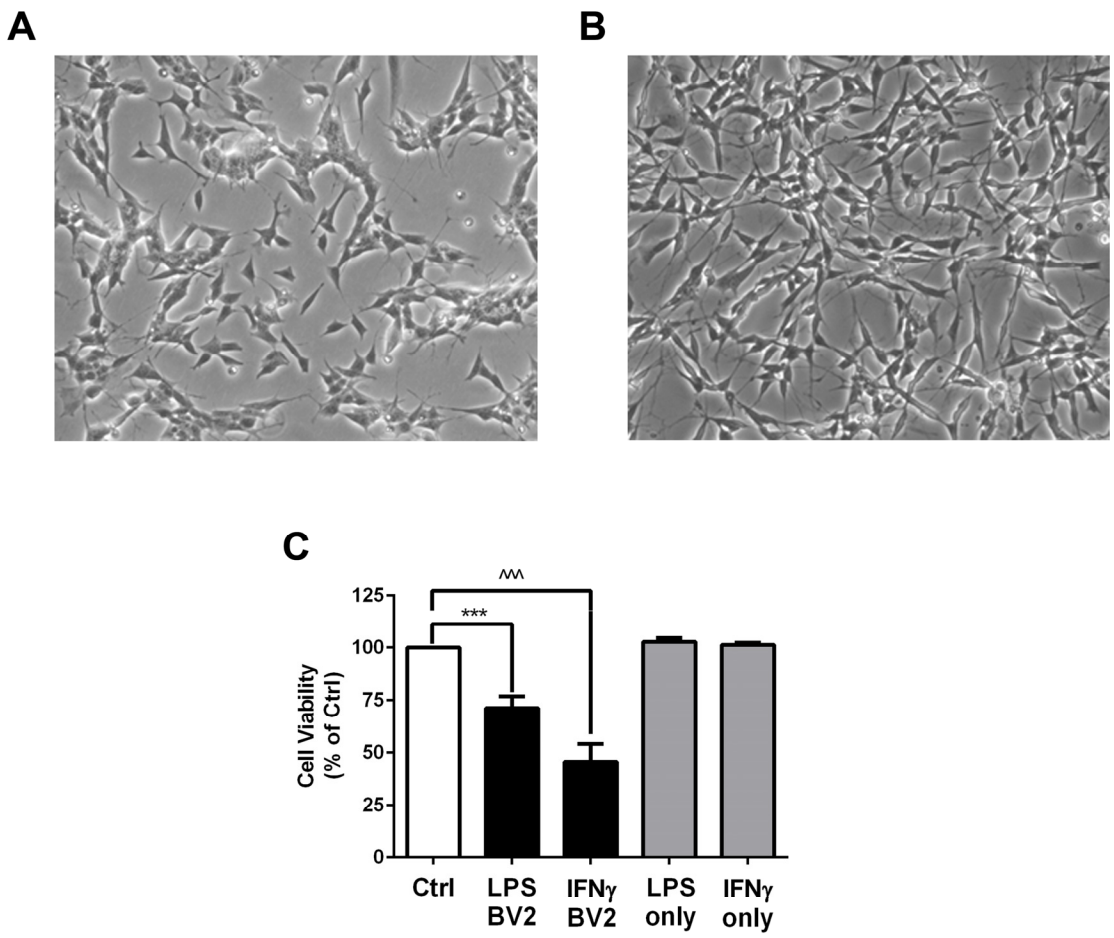

Figure 4.4

SH-SY5Y differentiation and microglia-induced neurotoxicity. (A, B) Representative bright field microscopic photography of SH-SY5Y cells prior to differentiation with and without 10 μM retinoic acid for 5 days. (C) Cell viability of SH-SY5Y cells after exposure to conditioned medium prepared from BV-2 cells treated with LPS or IFNγ for 24h, or with LPS and IFNγ in fresh culture medium for 48h. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, *** $P < 0.001$, ^^^ $P < 0.001$.

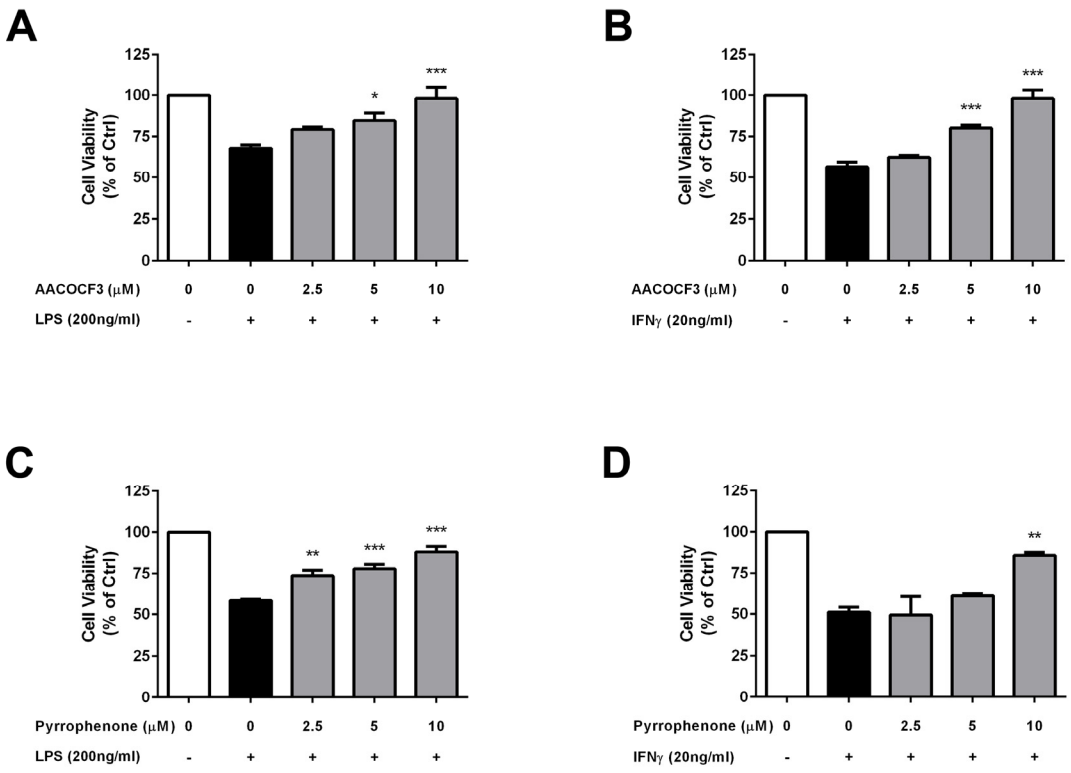

Figure 4.5

Microglia-induced neurotoxicity was attenuated by cPLA$_2$ inhibitors. BV-2 cells were serum-starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of cPLA$_2$ inhibitors: (A-B) AACOCF3 or (C-D) pyrrophenone. Cells were then stimulated with (A, C) 200ng/mL LPS or (B, D) 20ng/mL IFNγ for 24 hours, and conditioned medium is then transferred to SH-SY5Y culture. (A-D) Cell viability of SH-SY5Y cells after exposure to conditioned medium for 48h. Results were expressed as the mean ± SEM (n = 3) and analyzed by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to LPS/IFNγ alone.

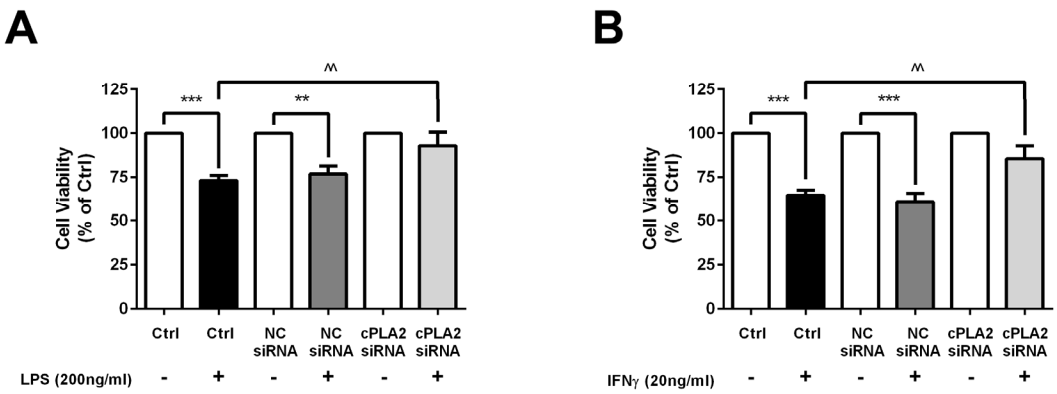

Figure 4.6

Microglia-induced neurotoxicity was attenuated by down regulation of cPLA$_2$ with siRNA. BV-2 cells were transfected with siRNA against cPLA$_2$ 24 hours before being stimulated with (A) LPS or (B) IFNγ. BV-2 cells were then stimulated with 200ng/mL LPS or 20ng/mL IFNγ for 24h and conditioned medium is then transferred to SH-SY5Y culture. (A-B) Cell viability of SH-SY5Y cells after exposure to conditioned medium for 48h. Results were expressed as the mean ± SEM (n = 3) and significant difference between the respective groups was determined by one-way ANOVA followed by Dunnett's post-tests, ** $P < 0.01$, *** $P < 0.001$, ^^$P < 0.01$.

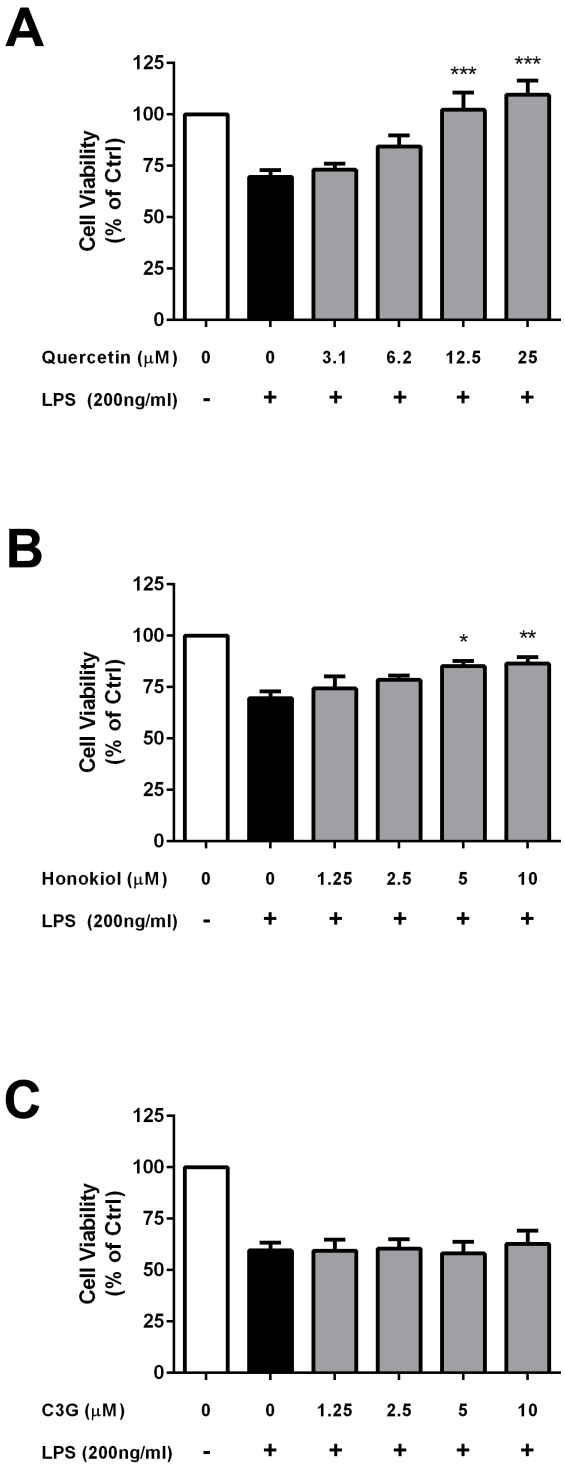

Figure 4.7

Microglia-induced neurotoxicity was attenuated by quercetin and honokiol but not C3G. BV-2 cells were serum-starved for 4 hours in serum-free DMEM. One hour prior to stimulation, cells were pretreated with the indicated concentrations of (A) quercetin, (B) honokiol, or (C) C3G. BV-2 cells were then stimulated with 200ng/mL LPS for 24h and conditioned medium is then transferred to SH-SY5Y culture. (A-C) Cell viability of SH-SY5Y cells after exposure to conditioned medium for 48h. Results were expressed as the mean ± SEM (n = 3) and analyzed by one-way ANOVA followed by Dunnett's post-tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to LPS alone.

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CHAPTER 5

DIETARY SUTHERLANDIA AND ELDERBERRY MITIGATE CEREBRAL ISCHEMIA-INDUCED NEURONAL DAMAGE AND ATTENUATE P47PHOX AND PHOSPHO-ERK1/2 EXPRESSION IN MICROGLIAL CELLS

ABSTRACT

Sutherlandia (*Sutherlandia frutescens*) and elderberry (*Sambucus* spp.) are used to promote health and for treatment of a number of ailments. Although studies with cultured cells have demonstrated anti-oxidative and anti-inflammatory properties of these botanicals, little is known about their ability to mitigate brain injury. In this study, C57BL/6J male mice were fed AIN93G diets without or with Sutherlandia or American elderberry for two months prior to a 30-minute global cerebral ischemia induced by occlusion of the bilateral common carotid arteries (BCCA), followed by reperfusion for three days. Accelerating rotarod assessment at 24 hours after BCCA occlusion showed amelioration of sensorimotor impairment in the mice fed the supplemented diets as compared with the ischemic mice fed the control diet. Quantitative digital pathology assessment of brain slides stained with cresyl violet at three days after ischemia/reperfusion (I/R) revealed significant reduction in neuronal cell death in both dietary groups. Immunohistochemical staining for Iba-1 demonstrated pronounced activation of microglia in the hippocampus and striatum in the ischemic brains three days after I/R, and microglial activation was significantly reduced in animals fed

supplemented diets. Mitigation of microglial activation by the supplements was further supported by the decrease in expression of p47phox, a cytosolic subunit of NADPH oxidase, and phospho-ERK1/2, a MAPK known to mediate a number of cytoplasmic processes including oxidative stress and neuroinflammatory responses. These results demonstrate neuroprotective effects of Sutherlandia and American elderberry botanicals against oxidative and inflammatory responses to cerebral I/R.

INTRODUCTION

Throughout human history, many natural products from plants have been suggested to promote human health and manage disease symptoms, and some have been developed into modern-day drugs. Studies in recent years have documented anti-oxidative and anti-inflammatory properties of fruits and vegetables and herbs, and indicate that some of these can maintain brain health during aging (Galli et al., 2002; Sun et al., 2008). It is important to understand the molecular mechanisms underlying their mode of action.

Sutherlandia [*Sutherlandia frutescens* (L.) R. Brown or *Lessertia frutescens* (L.) Goldblatt & J.C. Manning], also known colloquially as cancer bush, is widely used in southern African traditional and contemporary remedies for a variety of chronic ailments, including cancer, arthritis, digestive disorders, and diabetes, and more recently, behavioral symptoms of HIV/AIDS such as depression and anxiety (Mills et al., 2005; van Wyk and Albrecht, 2008). Studies with cell and animal models have demonstrated its

antioxidant and anti-inflammatory properties (Fernandes et al., 2004; Ojewole, 2004; Katerere and Eloff, 2005; Kundu et al., 2005; Faleschini et al., 2013; Jiang et al., 2014). While some evidence supports Sutherlandia's benefit for mitigating stress (Prevoo et al., 2004) as well as drug-induced seizures (Ojewole, 2008), little is known about its broader effects against neurodegenerative diseases and stroke. Results from a randomized, double-blind, placebo-controlled trial in healthy adults of consumption of Sutherlandia for 3 months showed it was well tolerated (Johnson et al., 2007).

Consumption of elderberry, including the North American subspecies (*Sambucus nigra* L. subsp. *canadensis* [L.] Bolli) has increased in recent years, mainly for its claimed ability to combat symptoms of common flu and other viral infections (Zakay-Rones et al., 1995; Monograph, 2005; Vlachojannis et al., 2010). Elderberries are widely cultivated in Europe, Asia, North Africa, and North America (Monograph, 2005). Elderberry fruit contains flavonoids and anthocyanins (Lee and Finn, 2007; Thomas et al., 2013) which are reported to have beneficial effects on human health, especially cardiovascular functions, anti-carcinogenic, anti-viral and anti-inflammatory effects (Prior and Wu, 2006; Zafra-Stone et al., 2007). Cyanidin-3-glucoside, one of the most common anthocyanins of berries, was shown to ameliorate ethanol-induced neurotoxicity in developing brains, and protect against focal cerebral ischemia in mice (Ke et al., 2011; Min et al., 2011). There is further evidence suggesting ability of berries to prevent age-associated oxidative stress and improve neuronal and cognitive functions in animal models (Galli et al., 2002). Despite the increasing interest regarding these secondary metabolites, little is known whether elderberries alleviate stroke damage.

Stroke is the second leading cause of death world-wide and is the primary cause of acquired disability in the United States (Davis and Donnan, 2012). Although the pathophysiology of ischemic damage is complex, extensive studies have focused on the underlying mechanisms of oxidative stress and inflammatory responses following ischemia/reperfusion (I/R) (Chen et al., 2011a; Chen et al., 2011b). Studies have demonstrated the role of NADPH oxidase and activation of the mitogen-activated protein kinase (MAPK) pathways in production of reactive oxygen species (ROS), and signaling events leading to mitochondrial dysfunction and activation of apoptotic pathways (Chen et al., 2011a; Yoshioka et al., 2011b). Among the various *in vivo* models of cerebral ischemia, the murine bilateral common carotid artery (BCCA) occlusion model has been documented to cause damage in the hippocampal and striatal neurons (Lin et al., 2000; Wang et al., 2005b; Yoshioka et al., 2011b). Previous studies with the gerbil global BCCA occlusion model demonstrated protective effects of botanicals such as curcumin and grape polyphenol extracts against neuronal cell death and glial cell activation in the hippocampal CA1 area (Wang et al., 2002; Simonyi et al., 2005; Wang et al., 2005b).

Both Sutherlandia and elderberry share the capacity to relieve oxidative stress and suppress inflammatory responses. In this study, the murine global cerebral ischemia model was used to demonstrate that dietary supplementation by Sutherlandia and American elderberry offer protection against ischemia-induced neuronal damage and glial cell activation and neurobehavioral dysfunctions.

MATERIALS AND METHODS

Materials

Antibodies used for immunohistochemical staining include: rabbit anti-p47phox antibody (sc-14015; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-phospho-ERK1/2 monoclonal antibody (9102; Cell Signaling, Beverly, MA). Cell type specific antibodies include: rat anti-CD11b (550274; BD Biosciences, San Jose, CA) and rabbit anti-Iba-1antibodies (019-19741; Wako BioProducts, Richmond, VA) for microglia, and rabbit anti-glial fibrillary acidic protein (GFAP) antibodies (G9269; Sigma-Aldrich, St. Louis, MO) for astrocytes. Secondary antibodies include goat anti-mouse IgG-Alexa488 (A11001), goat anti-rabbit IgG Alexa 488 (A110034), goat anti-mouse IgG Alexa fluor 594 (A11005), and goat anti-rat IgG Alexa fluor 594 (A11007; Life Technologies/Invitrogen, Carlsbad, CA).

Animals, diets and ischemia protocol

Adult male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) at age 8 weeks were housed 4/cage and maintained on a 12-hour light/dark cycle (lights on at 7:00 AM) with unrestricted access to food and water. Prior to surgical BCCA occlusion, mice were fed for two months with a nutritionally-complete experimental diet AIN93G with or without supplement of either 1% by weight of freeze-dried, ground Sutherlandia dried vegetative material or 2% by weight of freeze-dried, ground whole ripe fruit of American elderberry, based upon empirical estimates of mouse equivalents of human consumption (Wang et al., 2005c; Johnson et al., 2007) (see Table 1 for complete composition of the control and test diets). Sutherlandia vegetative material was purchased from Big Tree

Nutraceutical (Fish Hoek, South Africa) and stored at -20°C in an air-tight container in the dark. The elderberry fruits were harvested in 2010 from a germplasm repository in southwest Missouri (USA), and frozen in zippered plastic freezer bags. Berries were later de-stemmed and cleaned, lyophilized, and ground into a fine powder before addition to diets. A mixture of several American elderberry genotypes was used in this study. Botanical vouchers confirming the taxonomic identity of plants were deposited into the herbaria of the University of Missouri or the Missouri Botanical Garden (St. Louis, MO). Average food intake: 2.6 ± 0.05 gram/day/mouse; and average diet consumption: 0.106 ± 0.003 gram/gram body weight/day. Weekly monitoring of body weight indicated no differences in weight between any of the groups at any time during the course of the study. Approved animal protocols were obtained and all treatment steps were in accordance with University of Missouri and the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

For the study, mice were divided into four experimental groups: (1) sham animals with AIN93G control diet (Sham/CD, n=7), (2) BCCA occlusion-induced ischemia with AIN93G diet (Isch/CD, n=7), (3) BCCA occlusion-induced ischemia with AIN93G diet containing 1% Sutherlandia (Isch/SD, n=7), and (4) BCCA occlusion-induced ischemia with AIN93G diet containing 2% elderberry (Isch/ED, n=7). To conserve on animal numbers, sham operation was performed only on animals with the control diet. Animals were subjected to a transient global cerebral ischemia by BCCA occlusion as described previously with minor modifications (Lin et al., 2000; Chen et al., 2011a). To initiate the surgical protocol, mice were placed in a holding chamber and anesthetized with 4%

isoflurane, and continuous anesthesia during surgery was maintained with 1-1.5% isoflurane in 70% nitrogen and 30% oxygen with a face mask. During the surgery, rectal temperature was monitored and maintained at 37 ± 0.5°C with a thermostat-controlled heating pad. BCCA occlusion was accomplished by applying micro-aneurysm clips on both common carotid arteries for 30 minutes followed by release of the clips and a 3-day reperfusion. Re-establishment of blood flow was confirmed by direct observation. Sham operation animals were subjected to the identical surgical procedures except for application of micro-aneurysm clips. After surgery, animals were placed in cages above a heating blanket to maintain rectal temperatures above 36ºC for 1-2 hours with active monitoring.

Assessment of sensorimotor functions

Assessment of sensorimotor functions was carried out by the rotarod test as described previously with modifications (Simonyi et al., 2012). Two days prior to BCCA occlusion, mice were trained on the rotarod (Med Associates, St. Albans, VT) in the acceleration paradigm (4-40 rpm/5 minutes) for three trials each day with a 30-minute inter-trial interval (see Fig. 5.1A for the experimental design with BCCA occlusion set as Day 0). Latency is defined as the time spent on the accelerating rotating rotarod without falling off or gripping and spinning rather than walking. Pre-operative baseline values were obtained by determining average of the three best performances. Post-operative testing (3 trials) were performed 24 hours after I/R, and the means were used for calculation of rotarod performance. Latencies measured at 24 hours after I/R were expressed as % of performances reached before surgery.

Brain tissue processing, histochemical staining and assessment of neuronal damage

Three days after I/R, mice were euthanized with isoflurane and brains were sectioned for histochemical staining and assessment of neuronal damage using a well-established protocol as described previously (Cui et al., 2012; Hadass et al., 2013). Briefly, mice were transcardially perfused with 4% paraformaldehyde in 100 mM phosphate buffer and brains were dissected and preserved for 24 hours in the same buffer. Serial coronal sections (40 μm) were obtained with a vibrotome (VT1200S, Leica Microsystems, Inc., Bannockbum, IL). In most instances, a total of 150~160 40-μm tissue sections from each brain were collected into 24-well plates. Serial brain sections 200-μm apart were mounted on poly-L-lysine coated glass slides, and followed by staining with cresyl violet, a stain for Nissl substance in the cytoplasm of neurons commonly used for assessment of neuronal cell death.

Pathological assessment of histological specimens was carried out in an unbiased manner using a high-throughput digital pathology system. Briefly, whole slide images (WSI) of the cresyl violet-stained brain sections were obtained using an automatic multi-focus plane, high-throughput digital pathology system (Aperio ScanScope CS digital scanner, Vista, CA). Extent of neuronal damage in the brain sections was analyzed in a double-blind manner using the following criteria for the grading scale: 0: no observable neuronal damage; 1: damaged neurons populate 0~25% of area; 2: damaged neurons populate 25~50% of area; 3: damaged neurons populate 50~75% of area; and 4: damaged neurons populate >75% of area (Cui et al., 2012; Hadass et al., 2013).

Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was carried out on brain sections for astrocytes (with GFAP) and microglia (with Iba-1 and CD11b) (Hadass et al., 2013), as well as p47phox and phospho-ERK1/2 (p-ERK1/2). Briefly, fixed coronal sections from the area of interest were washed with phosphate buffered saline (PBS) and permeabilized with 1 % Triton X-100 in PBS for 30 min. Sections were incubated with Pro-Block (PBK125; ScyTek, Logan, UT) for 5 minutes to eliminate the need to match species with the fluorescence conjugated antibody, followed by 10% normal goat serum in 0.05% Triton X-100 in PBS for 60 min, and then overnight with 0.5% normal goat serum in 0.05% Triton X-100 in PBS containing the primary antibodies (GFAP, 1:500; CD-11b, 1:400; Iba-1, 1:1000; p47phox, 1:400; and p-ERK 1:500). The next day, sections were washed and incubated in 0.05% Triton X-100 in PBS containing the appropriate fluorophore-conjugated secondary antibodies (1:300; goat anti-mouse IgG-Alexa488, goat anti-mouse IgG-Alexa594, goat anti-rabbit IgG-Alexa488, and goat anti-rat IgG Alexa594; Life Technologies/Invitrogen, San Diego, CA) for 2 hours, and counterstained in a solution of Hoechst dye 33342 (1:1000; H-3570, Life Technologies/Invitrogen, San Diego, CA).

Fluorescence photomicrographs of the areas of interest were captured by a Leica DMI 6000B automated epifluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL) and the high magnification photomicrographs were processed using the AF6000 stitching program and intensity analysis with the ImageJ program. For quantification of immunofluorescence intensity, five representative areas in the striatum

(coordinated approximately to Bregma 0.78 mm) were selected bilaterally (as indicated in the Fig. 5.4A) and the microphotographic images of the immunostained brain sections were captured. All microphotographic images for examining each set of the experimental groups were taken under the same camera and microscope settings including the dimension, voxel size, and exposure parameters (intensity, exposure time and gain for each channel, as well as threshold values for black as 0, white as 255, and binning 1x1), except pERK which has threshold values from 99 to 255.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Results were analyzed by one-way ANOVA with Bonferroni's post-test (V4.00; GraphPad Prism Software Inc., San Diego, CA). Statistical significance was considered for $p<0.05$.

RESULTS

Dietary supplementation with Sutherlandia or elderberry ameliorated motor impairment in mice after transient global cerebral ischemia

A time line of the experimental protocol, showing feeding of mice at 2 months of age, pre-surgery and post-surgery rotarod tests and sacrifice for brain pathology is shown in Fig. 5.1A. In this experiment, sensorimotor functions in the sham, ischemia, and ischemia with Sutherlandia and elderberry diet groups were assessed using the accelerating rotarod paradigm. In the pre-ischemia rotarod tests, no differences were

observed in sensorimotor functions between controls and the different dietary groups (Sham/CD, 254.0 + 5.9; Isch/CD, 245.6 + 10.7; Isch/SD, 247.0 + 12.5; Isch/ED, 275.7 + 6.2, sec; p>0.05, One-way ANOVA). Rotarod performance was unchanged in the sham-operated group but was significantly impaired in the ischemia group assessed 24 hours after reperfusion (Fig. 5.1B). Consumption of the Sutherlandia or elderberry supplemented diets for 2 months ameliorated the sensorimotor deficits by prolonging rotarod latencies.

Consumption of Sutherlandia and elderberry diets decreased neuronal damage after I/R

Cresyl violet staining of brain sections revealed substantial damage in neuronal morphology after BCCA occlusion (Fig. 5.2A): while normal healthy neurons were round with pale-stained cytoplasm, many neurons in the ischemic regions in cortex, hippocampus and striatum were angular in shape with condensed cell bodies (see higher magnification insets in Fig. 5.2B, D and F, respectively). The zoomable WSI photomicrographs were acquired from approximately 30-35 serial tissue sections with a 200-μm interval per brain. Damage scoring (0-4, based on the criteria described in the Methods section) for cerebral cortex, hippocampus and striatum including thalamus and basal ganglia areas were carried out in a double blind manner using the web-based ImageScope program. Quantitation of the cellular damage in each region revealed that I/R resulted in severe neuronal damage in the hippocampus but those in the striatum/thalamus/basal ganglia area and the cortical regions were also damaged albeit to a lesser extent (Fig. 5.2B and C). Dietary supplementation with elderberry significantly decreased the neuronal damage in all the brain regions examined (Fig. 5.2C, E and G). To

a lesser extent, decrease in neuronal damage was also observed in these brain regions upon supplementation with Sutherlandia.

Immunostaining of microglial cells and astrocytes

Neuronal damage after I/R is often accompanied by increased neuroinflammatory responses including astrogliosis and microglial activation (Wang et al., 2006; Brennan et al., 2009; Chen et al., 2011b). We assessed these responses three days after I/R by immunostaining brain sections with Iba-1 for microglia and GFAP for astrocytes. Immunofluorescent analysis with Iba-1 revealed low immunoreactivity in sham controls but a robust and widespread increase in the ischemic brain, especially in the hippocampal and striatal regions (Fig. 5.3A, Isch/CD). Both Sutherlandia and elderberry diets attenuated the Iba-1 immunoreactivity in the cortex and striatum (Fig. 5.3A, Isch/SD and Isch/ED).

We also examined brain sections immunostained with GFAP as a marker for activated astrocytes. Immunoreactivity of GFAP was low in sham controls but increased after I/R. The pattern for GFAP immunoreactivity appeared to be more spread out and diffuse as compared with those stained with Iba-1 immunoreactivity (Fig. 5.3B).

Sutherlandia and elderberry consumption attenuated I/R-induced activation of microglia but not astrocytes in the striatum

Since the striatal region is more homogeneous, we selected five representative subregions for further examination of cell immunoreactivity and morphology (Fig. 5.4A).

Immunoreactivity of Iba-1 expressing microglial cells in the sham-operated group appeared mostly in the resting ramified form with small round cell bodies and thin processes (Fig. 5.4B), whereas those in the ischemic regions became amoeboid shape with irregular cell bodies and thick processes (see insets in Fig. 5.4B). By assessing average fluorescence intensity of Iba-1 in these striatal regions, significantly lower immunoreactivity of microglia in mice fed the Sutherlandia or elderberry diets was found as compared with the ischemia group on the control diet (Fig. 5.4C).

Similarly, fluorescence intensity of GFAP in the striatal regions indicated a significant increase in GFAP immunoreactivity after BCCA occlusion as compared to sham controls (Fig 5.4D). However, both Sutherlandia and elderberry diets did not attenuate GFAP immunoreactivity as compared to the ischemia group on the control diet (Fig. 5.6D).

Sutherlandia and elderberry consumption inhibited I/R-induced increases in p47phox expression in the striatum

A number of studies have demonstrated involvement of NADPH oxidase in ROS production during I/R (Wang et al., 2006; Chen et al., 2009). Double immunostaining of p47phox, an NADPH oxidase subunit, with GFAP for astrocytes and CD11b for microglial cells showed that p47phox immunoreactivity did not colocalize with the GFAP expressing astrocytes (Fig. 5.5A), but instead with the CD11b expressing microglia (Fig. 5.5B).

Quantitation of the p47phox immunoreactivity was carried out in the five selected areas in the bilateral striatal regions. A significant increase in p47phox immunoreactivity was observed in the striatum (Fig. 5.6A) as well as in the hippocampus (data not shown) of the ischemic brain at three days after I/R. Measurement of fluorescent intensity from different areas in the striatal and caudate/putamen region indicated that mice given either botanical diet had a significant decrease in p47phox immunoreactivity as compared with the ischemic group (Fig. 5.6B).

Sutherlandia and elderberry consumption inhibited I/R-induced phospho-ERK1/2 expression in microglial cells

Recent studies with cultured microglial cells demonstrated involvement of Extracellular Signal-Regulated Kinases 1/2 (ERK1/2) in the oxidative/nitrosative pathways associated with stimulation by lipopolysaccharide (LPS) and interferon gamma (IFNγ) (Chuang et al., 2013). Based on the observations of increased p47phox immunoreactivity and activated microglial cells in striatum after I/R, we further examined phospho-ERK1/2 expression in the ischemic brain sections and compared immunoreactivity with the groups supplemented with Sutherlandia and elderberry diets. Double immunostaining for phospho-ERK1/2 and microglial marker CD11b indicated increased phospho-ERK1/2 immunoreactivity colocalized with many microglial cells in the striatum at three days after I/R (Fig. 5.7A). Fluorescence intensity analysis showed a significant decrease in phospho-ERK1/2 immunoreactivity in both dietary groups as compared with the ischemia control group (Fig. 5.7B and 5.7C).

DISCUSSION

This study demonstrates that dietary Sutherlandia and elderberry mitigate behavioral deficits and pathology induced by BCCA occlusion in mice. These results agree with and extend our earlier studies using the Mongolian gerbil model demonstrating botanicals such as curcumin, apocynin, and grape polyphenols protect against ischemic damage (Wang et al., 2005a; Wang et al., 2005b; Wang et al., 2006; Wang et al., 2009). With the gerbil model, BCCA occlusion for 5 min causes extensive neuron cell death and glial activation in the hippocampal CA1 area four days after I/R (Wang et al., 2002). More recent studies by others using the murine model, and BCCA occlusion for 22 min resulted in damage in the hippocampal area as well as the striatal area (Yoshioka et al., 2011a). In the present study, we adopted the moderately severe murine BCCA occlusion model to investigate effects of dietary Sutherlandia and elderberry on I/R injury. BCCA occlusion for 30 min and followed by reperfusion for 3 days resulted in severe neuronal damage in the hippocampus, and sporadic cell death in the cortex as well as in the striatal/thalamus/basal ganglion regions. Using digital pathology and the 5 point scoring system on cresyl violet-stained brain sections, the results demonstrated dietary Sutherlandia and elderberry significantly mitigate the I/R-induced neuronal damage in all three brain regions. A behavioral test using the accelerating rotarod paradigm to monitor sensorimotor deficits of individual animals further showed that dietary supplementation of Sutherlandia and elderberry significantly ameliorated the I/R-induced motor/coordination deficits.

Cerebral ischemia not only causes damage to neurons, but also activates glial cells, both astrocytes and microglia. In previous studies with the gerbil model, a 5-min BCCA occlusion followed by reperfusion for 4 days led to prominent activation of astrocytes and microglial cells around the hippocampal CA1 region, where pyramidal neurons are extensively damaged (Wang et al., 2005a; Wang et al., 2009). With this murine model, we observed substantially greater activation of microglial cells at three days after a 30-min BCCA occlusion. The pattern of microglial cells activation reflects the areas where neurons are damaged. Alteration in microglial cell morphology towards the phagocytic form at this time after reperfusion is in agreement with the notion that microglia are actively responding to neuronal injury and cell death. The observation that mice consuming the Sutherlandia or elderberry diets showed significantly less microglial activation as compared to the ischemic brains of mice consuming control diet supports the capacity of these diets to mitigate neuronal damage and microglial activation.

With the BCCA occlusion model, a significant increase in astrocytes can be observed in the ischemic brain 3 days after I/R. Unlike the focal ischemia model where extensive astrogliosis is found in the penumbral area, astrocytes in the BCCA brain are more wide-spread in different brain regions. Furthermore, there is no significant difference in GFAP immunoreactivity comparing ischemic mice given the Sutherlandia or elderberry diets with control diets. These results further demonstrate effects of Sutherlandia and elderberry to protect ischemic damage through inhibiting neuron cell death and microglial cell activation. Although these results suggest an intimate

relationship between neuronal damage and activation of microglial cells, more studies are needed to better understand the mode of communication between these two cell types.

Increase in oxidative stress is an important factor in reperfusion injury; and several studies have implicated the involvement of NADPH oxidase as an important source of ROS (Chen et al., 2009; Yoshioka et al., 2011b). Although mechanisms for ROS produced by NADPH oxidase in neurons and glial cells are not well understood, ours and other's studies (Brennan et al., 2009) demonstrated rapid production of ROS in primary neurons (minutes) upon stimulation by the ionotropic glutamate receptor agonist (N-methyl-D-Aspartic Acid, NMDA) (Shelat et al., 2008); whereas production of ROS in microglial cells follows a delayed process in hours (Chuang et al., 2013). When botanicals such as honokiol and Sutherlandia extract were used to test the anti-oxidative effect on neurons (stimulated with NMDA) and microglial cells (stimulated with LPS), neurons were more sensitive to the anti-oxidative action than microglial cells (Chuang et al., 2013; Jiang et al., 2014). The role of NADPH oxidase in ROS production in neurons was demonstrated by using neurons from p47phox deficient mice which showed diminished response to ROS production in response to excitotoxic agents (Brennan et al., 2009). In the mouse model of global cerebral ischemia, an increase in p47phox immunoreactivity was observed in mouse striatum three days after I/R (Yoshioka et al., 2011b). In the present study, we further localize the I/R-induced increase in p47phox immunoreactivity to microglial cells. Again, the significantly lower expression of p47phox immunoreactivity in mice given the Sutherlandia and elderberry diets as

compared to that given the control diet is in agreement with the observation of decreased microglial activation.

It has been reported that I/R stimulates activation of MAPK pathways, in particular, the p38 MAPK and the Ras/MEK signaling (Nito et al., 2008), which are attributed to activation of the aquaporin-4 channel responsible for astrocyte swelling (Nito et al., 2012). Other studies also demonstrated upregulation of the MAPK/ERK1/2 pathway in brain after stroke (Sawe et al., 2008; Yenari et al., 2010). In fact, ERK1/2 is regarded as the most important member of the MAPK family capable of mediating a range of cellular responses, including motility, inflammation, and cell survival as well as cell death (Sawe et al., 2008). In our recent study with microglial cells, IFNγ not only stimulates the canonical JAK-STAT pathway but also the MAPK/ERK1/2 pathway, and in turn, phospho-ERK1/2 is linked to activation of a number of cytoplasmic proteins including p47phox of NADPH oxidase for ROS and iNOS for NO production (Chuang et al., 2013). Subsequently, inhibition of phospho-ERK1/2 by U0126 abrogated IFNγ-induced NO and ROS production in a dose-dependent manner (Chuang et al., 2013). Our recent study also demonstrated the capacity of Sutherlandia extracts to inhibit IFNγ-induced phospho-ERK1/2 and subsequently mitigate ROS and NO production in microglial cells (Jiang et al., 2014). Botanical polyphenols, e.g., the active ingredient of green tea, epigallocatechin-3-gallate, also attenuate NO production through down-regulation of ERK1/2 associated proteins including ALDH2, ANXA1 and LGALS1 in LPS-stimulated BV-2 microglial cells (Qu et al., 2014). Other studies also demonstrate a critical role of the MAPK/ERK pathway in neuronal excitation (Simon et al., 1984) and

MEK/ERK inhibitors mitigating brain damage in the stroke model (Wang et al., 2003; Wang et al., 2004; Gladbach et al., 2013). Results of this study further support the important role of phospho-ERK1/2 expression in microglial cells after I/R, and suppression in mice fed diets supplemented with Sutherlandia and elderberry.

Since Sutherlandia is widely used in southern Africa for symptoms of HIV/AIDS and elderberry dietary supplements are among top selling products in Europe and North America, these studies provide new insights into use of these herbs as neuroprotective agents. In summary, we have demonstrated significant protective effects of dietary elderberry and Sutherlandia against global cerebral ischemia-induced functional motor deficits and neuropathological changes, including neuronal cell death and microglial activation. Results further support the hypotheses that these botanicals exert beneficial effects against ischemic damage through suppression of oxidative and proinflammatory pathways in neurons and microglial cells. This study provides strong rationale to further investigate the active components and mechanisms of action and to determine whether their consumption ameliorates ischemic damage as well as neurodegenerative diseases.

	Control Diet		1% Sutherlandia Diet		2% Elderberry Diet	
Ingredient	g/kg of diet	% of total	g/kg of diet	% of total	g/kg of diet	% of total
Cornstarch	397.3	39.73	387.3	38.73	387.3	37.73
Casein	200.0	20.00	200.0	20.00	200.0	20.00
Dextrose	132.0	13.20	132.0	13.20	132.0	13.20
Sucrose	100.0	20.00	100.0	10.00	100.0	10.00
Fiber (cellulose)	50.0	5.00	50.0	5.00	50.0	5.00
Mineral mix (AIN-93)	35.0	3.50	35.0	3.50	35.0	3.50
Vitamin mix (AIN-93G)	10.0	1.00	10.0	1.00	10.0	1.00
L-cystine	3.0	0.30	3.0	0.30	3.0	0.30
Choline bitartrate	2.5	0.25	2.5	0.25	2.5	0.25
Soybean oil	70.0	7.00	70.0	7.00	70.0	7.00
Food dye (color varies)	0.2	0.02	0.2	0.02	0.2	0.02
Elderberry (whole ripe fruit)[1]	/	/	/	/	20	2.00
Sutherlandia (dried leaves)[2]	/	/	10	1.00	/	/
TOTAL	1000	100	1000	100	1000	100

[1] York and Bob Gordon cultivars (60:40 ratio) were harvested at the peak of ripeness, freeze-dried, then ground into a fine powder and mixed with an equal weight of corn starch to prevent clumping. The amount of this mix added to the diet was actually twice that shown in the table.

[2] Sutherlandia was sourced Big Tree Nutraceutical (21 First Avenue, Fish Hoek 7975, South Africa). Prior to its incorporation into the diet, the chopped leaves were grounded into a fine powder using a hand-held coffee bean grinder.

Table 5.1

Composition of the control AIN93G and diets supplemented with Sutherlandia and elderberry

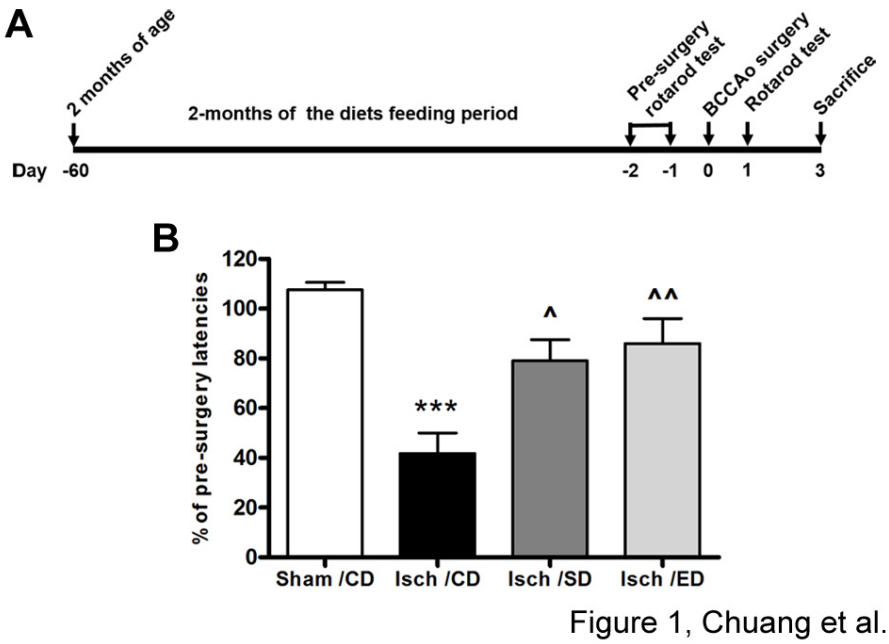

Figure 5.1

Supplementation of Sutherlandia and elderberry diets and assessment of motor coordination and balance functions in ischemic mice. (A) Experimental design for dietary feeding, rotarod assessment and BCCA occlusion (BCCAo). (B) Accelerating rotarod assessment reveals amelioration of behavioral deficits from transient global cerebral ischemia by dietary supplement of Sutherlandia and elderberry. Rotarod performance is expressed as percent of time the mouse can stay on the accelerating rotarod compared with pre-ischemia as the baseline values. Four groups of mice were divided into (1) AIN93G control diet (Sham/CD), (2) BCCA occlusion-induced ischemia with control diet (Isch/CD), (3) BCCA occlusion-induced ischemia + Sutherlandia diet (Isch/SD), and (4) BCCA occlusion-induced ischemia + elderberry diet (Isch/ED). Data are expressed as means ± SEM (n=7 for all groups). Statistical significance is denoted with *** p<0.001 (compared with Sham/CD); ^ p<0.05 and ^^ p<0.01 (compared with Isch/CD) by one-way ANOVA followed by Bonferroni's post-test.

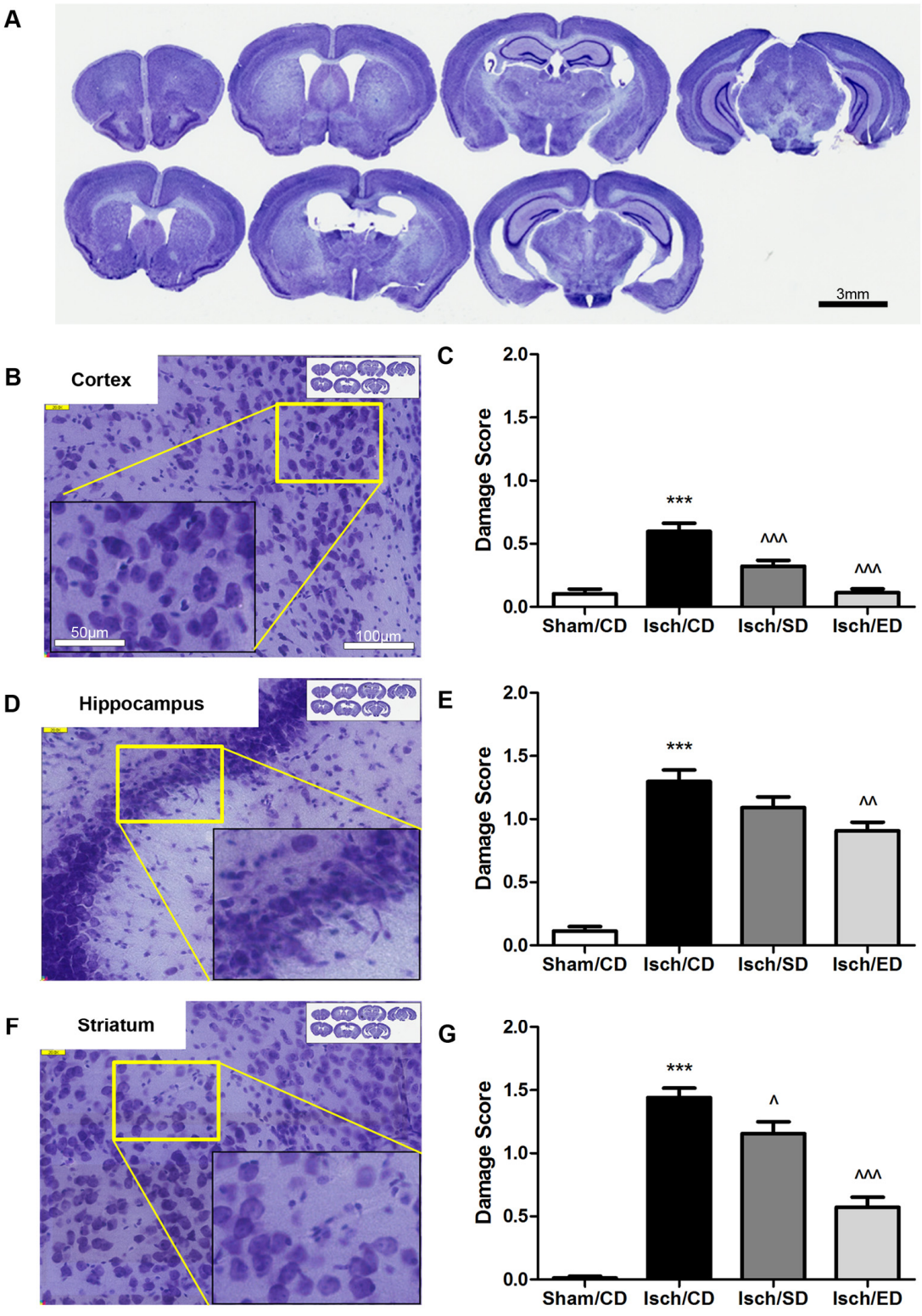

Figure 2, Chuang et al.

Figure 5.2

Consumption of Sutherlandia and elderberry diets ameliorates neuronal damage after transient global cerebral ischemia. (A) Representative photomicrographs of serial brain sections, each 960-µm apart; Scale bar = 3 mm. (B, D and F) Representative images of neurons from cortex, hippocampus and the striatal/ thalamus/basal ganglia areas; Scale bar = 100 µm, and 50 µm for the inset. (C, E and G) Graphical presentation of neuropathology scores in the respective brain regions (0 = no damage, 4 = maximal damage). Data are expressed as means ± SEM, where n=7 animals from each group. Statistical significance is denoted with *** p<0.001 (compared with Sham/CD); ^ p<0.05, ^^ p<0.01 and ^^^ p<0.001 (compared with Isch/CD) with one-way ANOVA followed by Bonferroni's post-test.

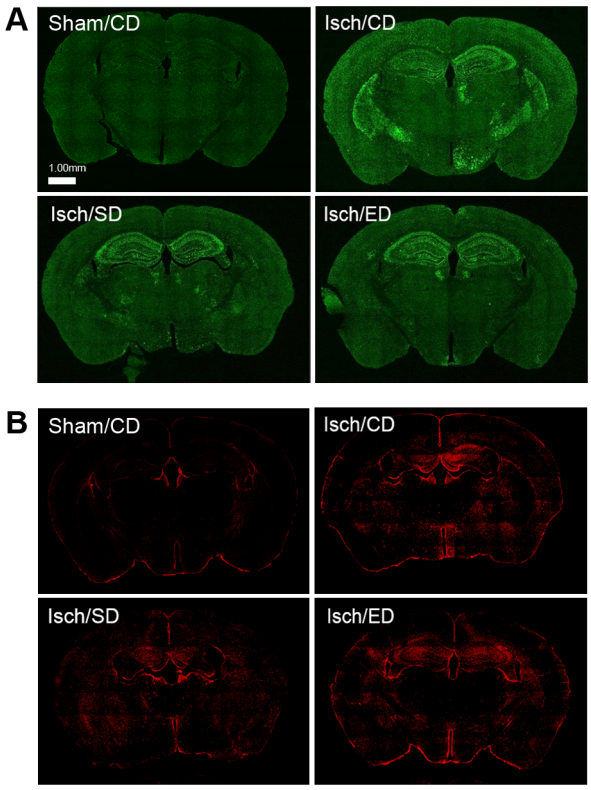

Figure 3, Chuang et al.

Figure 5.3

Brain sections immunostained with Iba-1 and GFAP. Microphotographs were generated by fluorescent microscope at resolution of 40x magnification for entire section regions, followed by automatic stitching of borders. (A) Representative brain fluorescent microscopic images of Iba-1 expression in the sham-operated mice with the control diet (Sham/CD), and the ischemic animals with the control diet (Isch/CD), Sutherlandia diet (Isch/SD), and elderberry diet (Isch/ED). (B) Representative whole-brain fluorescent microscopic images of GFAP expression in the sham-operated mice with the control diet (Sham/CD), and the ischemic animals with the control diet (Isch/CD), Sutherlandia diet (Isch/SD), and elderberry diet (Isch/ED). Scale bar = 1.00 mm in A and B.

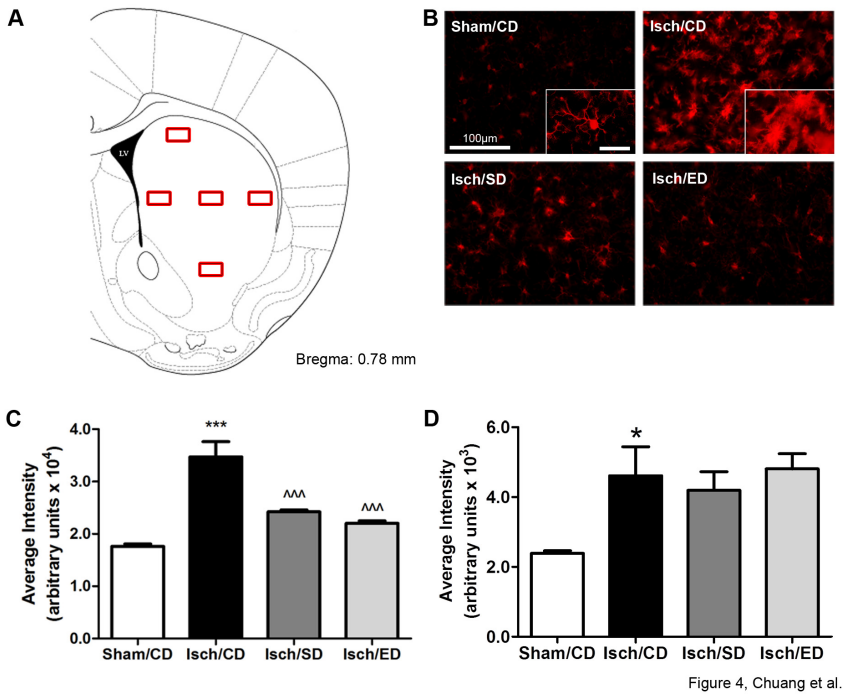

Figure 5.4

Consumption of Sutherlandia and elderberry diets attenuates activation of microglial cells but not astrocytes in the striatal regions after transient global cerebral ischemia. (A) Graphical illustration of the five representative areas selected bilaterally for captured intensity analysis. (B) Representative fluorescent microscopic images of Iba-1 immunoreactivity among all groups; Scale bar = 100 μm. Inset in Sham/CD panel shows representative cells of the ramified resting microglia, while inset in Isch/CD panel the amoeboid form of activated microglia; Scale bar = 25 μm. (C and D) Quantitation of the average fluorescent intensity for Iba1 (C) and GFAP (D) immunoreactivity from 5 areas in the striatum (n=5 for all groups). Data are expressed as means ± SEM. Statistical significance is denoted with * $p<0.05$, *** $p<0.001$ (compared with Sham/CD); and ^^^ $p<0.001$ (compared with Isch/CD) by one-way ANOVA followed by Bonferroni's post-test.

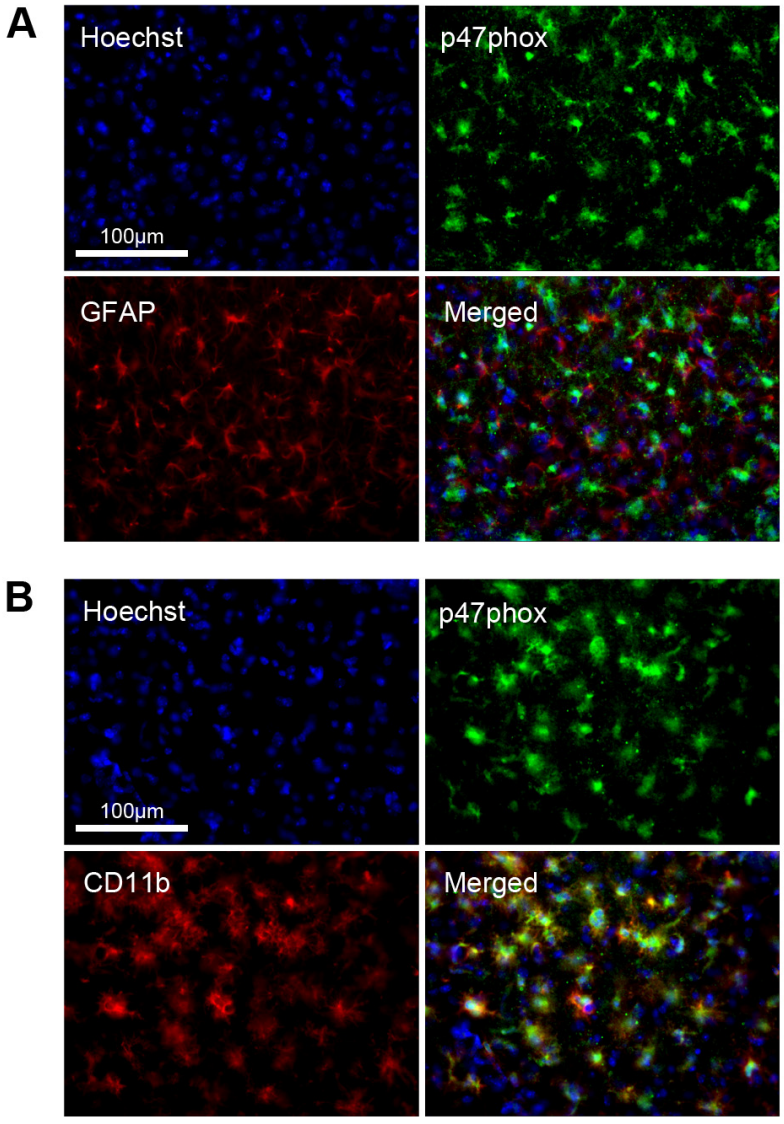

Figure 5, Chuang et al.

Figure 5.5

p47phox expression colocalized with microglia, and not astrocytes, in the striatum at 72-hours after ischemia/reperfusion. (A) Fluorescent microscopic image of p47phox (green) and GFAP (red) staining showing no colocalization between the p47phox and astrocytes. (B) Fluorescent microscopic image of p47phox (green) and CD11b (red) staining showing co-localization between the p47phox and microglia; Scale bar = 100 μm in A and B.

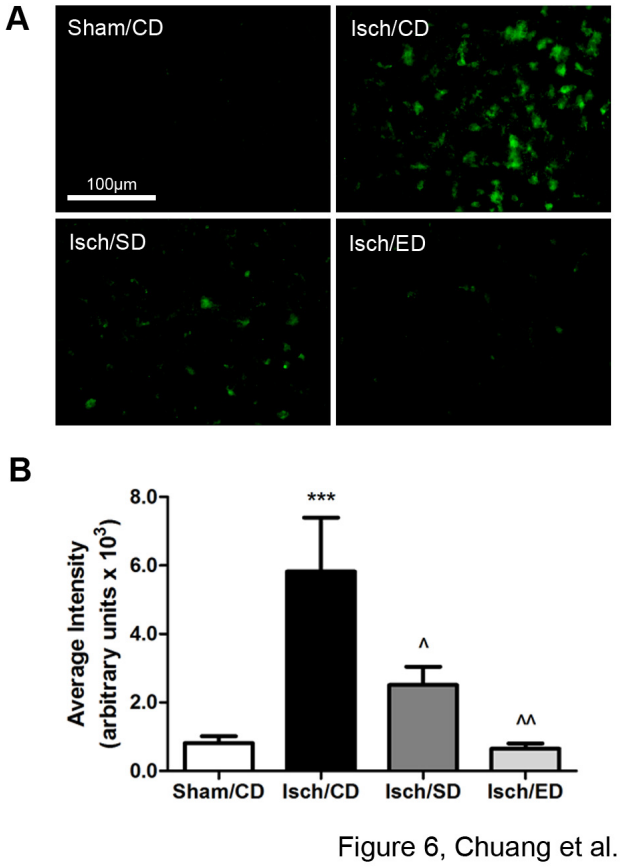

Figure 6, Chuang et al.

Figure 5.6

Immunostaining showing increase in p47phox expression in ischemic brain and attenuation by consumption of Sutherlandia and elderberry diets. (A) Representative fluorescent microscopic images of p47phox expression among four groups: Sham/CD, Isch/CD, Isch/SD and Isch/ED; Scale bar = 100 μm. (B) Graphical presentation of the average fluorescent intensity from five areas of interest (n=5 for all groups). Data are expressed as means ± SEM. Statistical significance is denoted with *** $p<0.001$ (compared with Sham/CD); ^ $p<0.05$ and ^^ $p<0.01$ (compared with Isch/CD) by one-way ANOVA followed by Bonferroni's post-test.

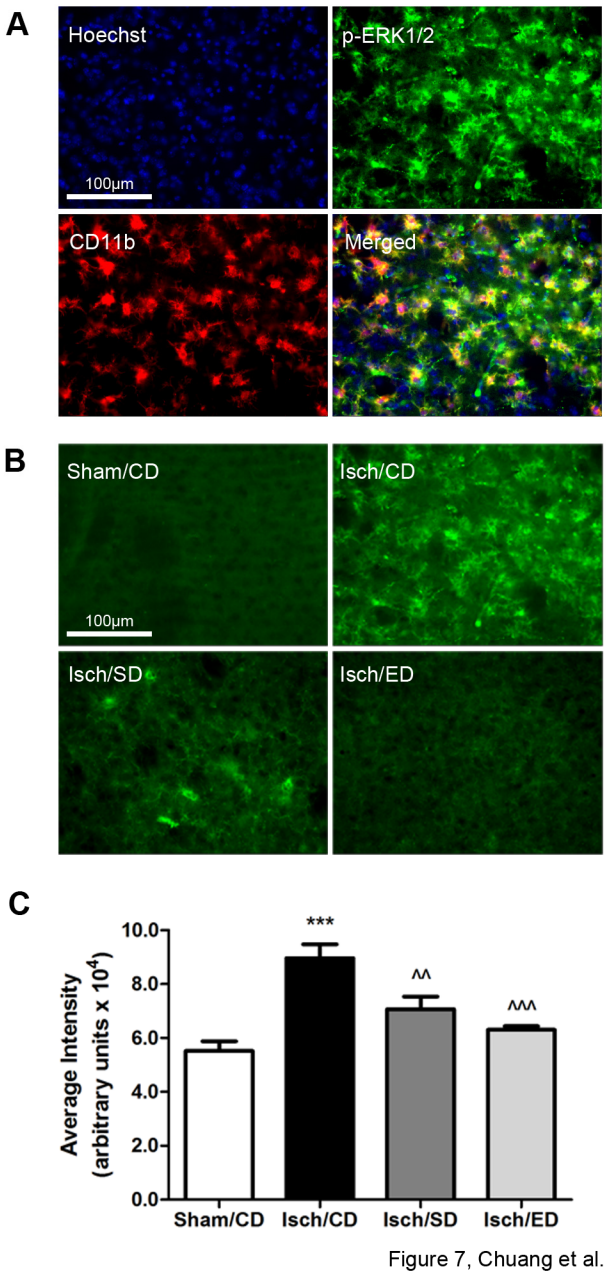

Figure 5.7

Increase in phospho-ERK1/2 expression in ischemic brain and attenuation by consumption of Sutherlandia and elderberry diets.

The increased phospho-ERK1/2 (p-ERK1/2) expression is colocalized with microglia. (A) Fluorescent microscopic image of p-ERK1/2 (green) and CD11b (red) staining showing co-localization between the p-ERK1/2 and microglia in the Isch/CD condition. (B) Representative fluorescent microscopic images of p-ERK1/2 expression among all groups. Scale bar = 100 μm in A and B. (C) Graphical presentation of the average fluorescent intensity from 5 areas of interest (n=5 animals were selected randomly for 7 in all groups). Data are expressed as means ± SEM. Statistical significance is denoted with *** $p<0.001$ (compared with Sham/CD); ^^ $p<0.01$ and ^^^ $p<0.001$ (compared with Isch/CD) with by one-way ANOVA followed by Bonferroni's post-test.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Through a series of studies, we have made important and novel findings regarding the role of microglia in neurological diseases, as well as advanced the understanding of signal transduction pathways for microglia activation. These studies also demonstrated the role of cPLA2 in signaling pathways of inflammation, as well as the effects of botanical polyphenols and botanical extracts to mitigate oxidative and inflammatory responses in microglial cells. Important findings are discussed as follows:

1. Role of ERK1/2 in activation of ROS from NADPH oxidase

In chapter 2, we first showed the importance of ERK1/2 phosphorylation and activation in the pathways for production of ROS and NO. We demonstrated involvement of p-ERK1/2 in the assembly of cytosolic NADPH oxidase subunits, such as p47phox, and their translocation to the membrane to form the active NADPH oxidase complex. We also validated NADPH oxidase to be the major source of ROS production in BV-2 microglial cells upon activation with LPS or IFNγ. Arguably, the method of validation was limited to use of pharmacological inhibitors in this study, which may result in non-specific enzyme inhibition. Future tools of validation may include knockdown/knockout of NADPH oxidase subunits. Also, while it is possible that ERK1/2 can contribute to the phosphorylation and activation of NADPH oxidase subunits, it would be interesting to

examine a direct role of this MAPK for the phosphorylation of p47phox and p67phox in BV-2 microglial cells after LPS/IFNγ stimulation.

2. Role of ERK1/2 in mediating cross-talk between LPS and IFNγ pathways

A novel finding in our studies is the recognition that LPS and IFNγ can independently stimulate oxidative and inflammatory responses in BV2 microglial cells. Since transcriptional synthesis of iNOS typically requires promotors from LPS/NF-κB and IFNγ/JAK-STAT pathways, these results suggest the presence of a cross-talk mechanism between the LPS and IFNγ pathways. To this end, our results provided evidence for the involvement of p-ERK1/2 in mediating the cross-talk. Although not studied in detail, similar phenomenon appears to occur in other immune cells, such as macrophages (Lei, Browning et al. 2015).

3. Honokiol and magnolol mitigate LPS- and IFNγ-mediated oxdative and inflammatory responses in primary neurons and BV-2 microglial cells

Polyphenols from Magnolia bark, including honokiol and magnolol, are known to offer medicinal value for *in vitro* and *in vivo* models of neurodegenerative diseases (Watanabe, Watanabe et al. 1983, Maruyama, Kuribara et al. 1998, Liu, Hattori et al. 2005, Xu, Yi et al. 2008, Chen, Lin et al. 2011). In our study, we investigated the effects of honokiol and magnolol on oxidative and inflammatory responses in primary cortical neurons (stimulated by NMDA) and microglial BV-2 cells (stimulated by LPS and IFNγ) (Chuang, Chan et al. 2013). Our results showed that these magnolia polyphenols are not only effective in inhibiting NMDA-induced neuronal superoxide production, but also

mitigate LPS- and IFNγ-induced ROS production in microglial cells. In addition, this study also demonstrated the involvement of ERK1/2 in mediating cross-talk for induction of iNOS, which corresponded to the ability of magnolia polyphenols to inhibit IFNγ-induced pERK in microglial cells. This study provided an example protocol for the Sun lab to subsequently test other botanical compounds and extracts e.g., studies on Sutherlandia and elderberry extracts and their active components (Jiang, Chuang et al. 2014, Simonyi, Chen et al. 2015).

Studies with elderberry extract and its polyphenol component demonstrated differences in potency for the anti-inflammatory effects among different polyphenols, e.g., quercetin, cyanidin, and other flavonoids with similar structure (Simonyi, Chen et al. 2015). In this study, quercetin was 10-fold more potent than cyanidin in inhibiting LPS-induced NO production in microglial cells. Besides in berries, quercetin is also present in other fruits including apples, in hot peppers and onions. The unique property of quercetin was being recognized by other investigators and efforts have been made to develop this nutraceutical as a potential therapeutic agent (Shaik, Castellani et al. 2006, Cai, Mazzoli et al. 2009, Simonyi, Chen et al. 2015, Sun, Chen et al. 2015). Besides inhibition of LPS-stimulated NO, quercetin (but not cyanidin) was also shown to stimulate the antioxidant pathway involving Nrf2/ARE and synthesis of phase II antioxidant and detoxifying enzymes (Sun, Chen et al. 2015). Unveiling the mechanism of polyphenols to down-regulate the NF-κB inflammatory pathway and up-regulate the Nrf2 antioxidant pathway has been the subject of intense studies recently in Sun Lab.

4. cPLA2 plays a crucial role in microglial inflammatory response

Prior studies in the Sun lab have focused on phospholipases A2, a group of enzymes that contributes to the release of arachidonic acid which is the key mediator for synthesis of prostaglandins and leukotrienoids by COXs and LOXs (Sun, He et al. 2012, Sun, Chuang et al. 2014). Among the different groups of PLA2, activation of cPLA2 has been implicated in a number of neurological diseases including stroke, AD and PD. Besides a calcium-binding domain that is required for its activity, this molecule is shown to consist of multiple phosphorylation sites, including the Ser-505 residue which can be phosphorylated by MAPKs. Aside from the established role for cPLA2 in systemic inflammation (Shmelzer, Haddad et al. 2003, Leslie 2015), there is increasing recognition regarding its role on diseases associated with neuro-inflammation. In chapter 3, we observed that microglial BV-2 cells have considerable expression of cPLA2, and that LPS and IFNγ both stimulate phosphorylation of cPLA2 through activation of ERK1/2. With this finding, a number of stringent validation efforts, including the use of genetic knockouts, knockdowns, and pharmacological inhibition against cPLA2, were carried out to establish the role for cPLA2 in primary and immortalized microglial cells. In agreement with the hypothesis that cPLA2 is crucial for LPS and IFNγ to induce ROS and NO, microglia isolated from cPLA2-/- brain showed limited ability to respond to LPS and IFNγ in induction of ROS and NO.

This study also included effort to isolate and characterize primary microglial cells from mouse brain using the protocol provided by Milteny Biotech (Chuang et al., 2015). Similar to BV-2 microglial cells, primary microglia also respond to LPS and IFNγ

resulting in the increase in the phosphorylation of ERK1/2 and cPLA2, as well as the subsequent release of prostaglandin E2 (PGE2). A novel observation of this study provided evidence that LPS- and IFNγ-induced NO and ROS production were not altered by the COX-1/2 inhibitor but were suppressed by the LOX-12 and LOX-15 inhibitors instead. These results provided new insights into the role of LOX-12/15 in bridging the release of arachidonic acid and the downstream production of ROS and NO.

Our study is probably the first to validate cPLA2's role within the microglial activation pathway through genetic knockout. We regarded this an important finding, providing a new target of intervention and offering insight into why cPLA2 inhibition may exert beneficial effects in neurodegenerative and neuroinflammatory diseases. Indeed, there are good examples whereby inhibitors for cPLA2 can mitigate brain injury and neurological diseases such as multiple sclerosis (Vana, Li et al. 2011), stroke damage (Zhang, Barasch et al. 2012), and spinal cord injury (Liu, Deng et al. 2014). While the discovery of cPLA2 and release of arachidonic acid in neuroinflammation may not come as a surprise to many, as its role is well-established in macrophages outside the central nervous system, our results unveiling the role of LOX-12/15 added new insights to the complexity of microglial inflammation and possible pharmacological agents for suppressing the oxidative and inflammatory responses. Obviously, more validation studies are warranted to investigate how LOX-12/15 contributes to the activation pathways in microglia. Our results also provide a possible explanation as to why some clinical trials for NSAIDS against COX-1/2 failed against treatment for

neurodegenerative diseases, and suggest an additional approach to target LOX inhibitors instead (Lee, Choi et al. 2013, Bader, Martini et al. 2015, Lim, Wenk et al. 2015).

5. *Activated microglials cells exert damaging effects on neurons and neurotoxic effects are mitigated by cPLA2 inhibition and botanical antioxidants*

In Chapter 4, we displayed the utility of an *in vitro* neuron-microglia interaction model to screen effects of botanical compounds in mitigating microglial activation. Our data show that botanical polyphenols, such as honokiol and quercetin which are known to suppress LPS-induced NO production, can also suppress phosphorylation of cPLA2. In order to demonstrate whether deleterious effects resulting from activated microglial cells may alter neuronal viability, a study was designed in which the conditioned medium from BV2 microglial cells was applied to differentiated SH-SY5Y neuronal cells. Consistent with our hypothesis, compounds that suppress microglial activation and inhibit cPLA2 phosphorylation, such as quercetin and honokiol, also could suppress microglia-induced neuronal cell death.

The above *in vitro* model provided a simple way to test the effectiveness of botanical compounds in mitigating microglial activation and subsequent neuronal damage. While our experiments provided a crude assessment of neuronal cell death, this is only the beginning towards future studies to establish protocols for high-throughput screening of botanical compounds. Future studies are needed to test the effects of botanicals on primary neurons and to identify the separate pathways and individual cytotoxic products responsible for toxicity.

In the attempt to show toxic effects of conditioned medium from activated microglial cells, future experiments may include testing a condition in which cultured medium containing the pro-inflammatory cytokines or LPS is replaced with flesh media after exposing microglia for 1-3 h. By removing LPS after brief exposure (sufficient time to activate the transcriptional pathways), cytotoxic compounds in the conditioned media will be limited to those released after stimulation of microglia.

6. *Dietary supplements of elderberry and Sutherlandia protect cerebral ischemia damage*

In chapter 5, an *in vivo* study was carried out to demonstrate the effects of dietary supplements of elderberry and Sutherlandia on neuronal cell death and microglial activation in an experimental model of cerebral ischemia induced by transient occlusion of the bilateral common carotid arteries (BCCA). In this study, mice were supplemented with the elderberry or Sutherlandia diet for 2 months prior to ischemic insult. Results showed partial protection of ischemic pathology, as well as alleviation of behavioral motor deficits in mice supplemented with both botanical diets. Consistent with *in vitro* experiments showing active components of elderberry and Sutherlandia extracts to mitigate oxidative and inflammatory responses in activated BV-2 microglial cells, this *in vivo* study also demonstrated evidence of suppressed microglial cells (but not astrocytes) in the ischemic brains of animals given the botanical diets. Furthermore, inhibition of activated microglial cells was associated with a significant decrease in the expression levels of phospho-ERK1/2 and p47phox, both markers of activation and oxidative stress.

Although the cause and effect relationship between prevention of neuronal cell death and suppression of microglial activation cannot be clearly delineated because the study used only a single time-point (3 days after ischemia/reperfusion), the protective effects of the botanical diets were evident and undeniable. Nevertheless, this study provided a protocol whereby investigation for other botanicals or pharmacological inhibitors can be carried out in an orderly manner. The ability to focus on microglial cells in specific brain areas also allow future studies to test temporal changes of these cells, e.g., expression of genes or proteins associated with the M1 pro-inflammatory and the M2 anti-inflammatory phenotypes.

Despite the favorable effect of botanicals demonstrated in this study, there exist a few limitations to the translational applicability of the study clinically relevant stroke. It should be noted that the BCCAO model, despite being a valid cerebral ischemic model, is not a representative clinical model. A typical ischemic stroke in clinical setting is normally confined to the area defined by the distribution of blood supply from specific cerebral vessels. On the other hand, BCCAO relies on restricting blood supply to the entire brain. As such, it is by definition a model for chronic global hypo-perfusion and heart failure. Despite the difference in pathophysiology of the models, the process of primary and secondary neuronal cell death is similar, thus making it a relatively valid model to investigate compounds that can prevent ischemic neuronal damage. Therefore, validation using a more clinically relevant model, such as the MCAO model, may be warranted in future studies. Also, initial focus of this experiment has been centered on preconditioning of the central nervous system to prevent ischemic damage. Considering

that microglia play a significant role in the secondary neuronal damage after ischemic attack, it would be of interest to investigate whether administration of these extracts or active compounds after stroke may result in decreased pathology of the brain. In addition, the MCAO model is probably more convenient to test the M1/M2 microglia phenotypes (Patel, Ritzel et al. 2013, Habib, Slowik et al. 2014, Pan, Jin et al. 2015).

In order to better understand the neuronal-glial relationship, future experiments may include a time-course study, e.g., examining neuronal damage at short times (such as 12 and 24 h) and microglial pathology at longer times (such as 1, 3, 5 and 7 days). A detailed time course study may allow further investigation of the morphology and markers for the M1 and M2 microglial phenotypes, and assessment of whether the botanicals play a role in suppressing the cytotoxic M1 and promoting the transformation of the neuro-protective M2 phenotype (Tang and Le 2015).

Finally, studies performed by others have shown consumption of Sutherlandia and elderberry to be safe in human. However, there is increasing realization that botanical extracts, due to their unknown and complex compositions, may interact with drugs. For example, supplementation of Sutherlandia A may suppress interaction with isoniazid for the prevention and treatment of tuberculosis (Johnson, Syce et al. 2007). While our preliminary *in vivo* data demonstrated favorable effect of Sutherlandia and elderberry diet in preventing cerebral ischemic damage in mice, it is important to investigate whether the same effect can be translated and observed in larger animals and ultimately human

patients. Similar studies can be extended to looking at TBI as well as other neurodegenerative diseases including PD and AD.

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VITA

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