THE ROLE OF PHOSPHOLIPASE A_2

IN AMYLOID β UPTAKE

BY MICROGLIA

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Master of Science

By

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

THE ROLE OF CPLA$_2$ IN AMYLOID BETA UPTAKE BY MICROGLIA

presented by Li Dong,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor James Lee

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

$AACOCF_3$	Arachidonyl trifluoromethyl ketone
Aβ	Amyloid Beta
AD	Alzheimer's disease
BCA	Bicinchoninic Acid Assay
BEL	Bromoenol Lactone
β-ME	2-Mercaptoethanol
BSA	Bovine serum albumin
$cPLA_2$	Cytosolic phospholipase A_2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FBS	Fetal Bovine Serum
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HRP	Horseradish Peroxidase
lFNγ	Interferon-γ
$iPLA_2$	Ca-independent phospholipase A_2
LPS	Lipopolysaccharides
MAFP	Methyl Arachidonyl Fluorophosphonate
MAPK	Mitogen-activated protein kinases
$p\text{-}cPLA_2$	Phospho-cytosolic phospholipase A_2

PS	Penicillin/Streptomycin
PFA	Paraformaldehyde
RIPA buffer	Radio Immunoprecipitation Assay buffer
$sPLA_2$	Secretory phospholipase A_2
TBS	Tris-buffered saline
TBST	Tris-buffer saline containing 0.1% (v/v) Tween 20
TRL2	Toll-like receptor 2

ABSTRACT

The accumulation of amyloid-β (Aβ) is a key characteristic of Alzheimer's disease (AD). Microglia are the principle macrophages in the brain and are known to internalize Aβ, however the phagocytic function is impaired as AD progresses. Cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) are two major groups of PLA_2s that are involved in modulating membrane properties, intracellular trafficking and the cellular inflammatory response. Here, we study the role of $cPLA_2$ and $iPLA_2$ in the uptake of $A\beta_{1-42}$ by microglia in vitro. We found that the uptake of $A\beta_{1-42}$ was rapid (<15 minutes) and remained unchanged up to 60 minutes. Also, inhibition of $cPLA_2$ greatly reduced $A\beta_{1-42}$ uptake while increasing $cPLA_2$ activation did not affect $A\beta_{1-42}$ uptake. $iPLA_2$ appears to reduce the rate of $A\beta_{1-42}$ uptake, but had no influence on the uptake level after 30 minutes. Furthermore, the incomplete depletion of $A\beta_{1-42}$ occurred within 5 minutes after uptake, with no detectable depletion occurring in the following 60 minutes. $cPLA_2$ and $iPLA_2$ are not involved in intercellular processing of $A\beta_{1-42}$. Instead, the results suggest the $A\beta_{1-42}$ undergoes processing in the lysosome to reduce the intercellular presence of $A\beta_{1-42}$. This study highlights the fact that microglia participate in $A\beta_{1-42}$ clearance and depletion via $cPLA_2$ endocytosis.

CHAPTER 1 INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a debilitating neurodegenerative disease that primarily develops among individuals 65 years and older. AD research began approximately a century ago with Dr. Alois Alzheimer who first described plaque deposits (later named senile plaques) and neurofibrillary tangles as two major pathological markers of AD (Alzheimer, 1907). Currently, AD is the most common form of dementia affecting approximately 5.2 million Americans in 2014 and these numbers are expected to increase as the aging population grows (Fargo and Bleiler, 2014). The symptoms of AD include memory loss, decline of intellectual function and changes in personality and behavior which become severer as the disease progresses over time. After decades of research the precise pathogenesis is still unclear with no known cure or effective treatment for AD. While the pathogenesis remains unknown research has uncovered a variety of effects occur in AD. Other than senile plaques and neurofibrillary tangles as histologic findings, changes including oxidative stress, inflammation, metal ion dysregulation, cell cycle dysregulation, tau phosphorylation have been reported (Webber et al., 2005).

1.2 Amyloid β peptide

In 1984 Glenner and Wong identified the amyloid beta peptide (Aβ) as a core protein in senile plaques that consisted of 39-43 amino acids (Glenner & Wong,

1984; Masters et al., 1985), and was generated from the abnormal proteolytic cleavage of amyloid precursor proteins (APPs) (Kang et al., 1987; Sisodia, Koo, Beyreuther, Unterbeck, & Price, 1990). Hardy and Higgins proposed the amyloid cascade hypothesis in 1992 which was universally accepted as the primary hypothesis for AD pathogenesis for many years (J. A. Hardy & Higgins, 1992). They proposed that the deposition of Aβ from the mismetabolism of APP initiated AD by forming senile plaques and neurofibrillary tangles, eventually causing the neuron and organism death (J. Hardy & Allsop, 1991; J. A. Hardy & Higgins, 1992). As more Aβ research became available over the last few decades the hypothesis has been modified, but the main premise that Aβ initiates AD pathogenesis remains the prevailing hypothesis.

The sequential cleavage of APP by β- and γ-secretase generates Aβ that can vary from 39-43 amino acids in length at the C-terminal due to the imprecise cleavage by γ-secretase (Iwatsubo et al., 1994; Iwatsubo, Saido, Mann, Lee, & Trojanowski, 1996). Interestingly, cleavage by α- and γ-secretase leads to the production of $sAPP_{\alpha}$ and the non-amyloidogenic pathway (Citron, Teplow, & Selkoe, 1995; Haass, 2004; Tian, Crump, & Li, 2010). The most common isoforms of Aβ are the 40-residue peptide ($A\beta_{1-40}$) and the 42-residue peptide ($A\beta_{1-42}$). Aβ can self-associate to form various aggregates including monomers, oligomers, protofibrils, fibrils and plaques. $A\beta_{1-42}$ was found to aggregate more rapidly than $A\beta_{1-40}$ (Kim et al., 2007). The correlation between senile plaques and neurotoxicity or cognitive deficits is tenuous (Giannakopoulos et al., 2003).

Recent evidence suggests that soluble Aβ oligomers are more neurotoxic in vitro and in vivo than larger aggregates (fibrils and plaques), especially the $A\beta_{1-42}$ form (Haass and Selkoe, 2007; Kuo et al., 1996; Shankar et al., 2008) which directly correlates with cognitive dysfunction (Cleary et al., 2005; McLean et al., 1999; Tomic et al., 2009). Since Aβ accumulation and/or aggregation directly correlates with AD progression, further investigations into the natural mechanism(s) of Aβ clearance should be a priority.

1.3 Microglia and AD

Microglia are the major resident macrophages in the brain, which serve as the main immune defense against host-derived waste and invading aliens in the CNS. In the AD brain, activated microglia were found to accumulate around senile plaques (Giulian et al., 1995), which indicated that accumulation of Aβ elicits an immune response. Further research has shown that microglia internalize Aβ in vitro (Ard, Cole, Wei, Mehrle, & Fratkin, 1996; Kopec & Carroll, 1998; Shaffer et al., 1995) as well as in vivo (Takata et al., 2007; Weldon et al., 1998). Krabbe *et al.* found that under Aβ burden, phagocytic activity of microglia was impaired (Krabbe et al., 2013), however the mechanism remains unclear. It is known that Aβ can trigger various inflammatory responses in microglia and these inflammatory mediators may facilitate Aβ clearance.

1.4 Phospholipase A_2 family

Phospholipase A_2 (PLA_2) is a family of enzymes that hydrolyzes phospholipids at the sn-2 position to produce fatty acids and lysophospholipids. PLA_2s are

categorized into three major groups: cytosolic phospholipase A_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) (Murakami & Kudo, 2002), multiple isoforms of which are present in the CNS. PLA_2s and their product, fatty acids, have been implicated in the pathologies of AD in a variety of capacities which include: inflammatory mediators, oxidative agents, calcium homeostasis and membrane fluidity (Farooqui & Horrocks, 2004; Sun, Xu, Jensen, & Simonyi, 2004). Furthermore, $iPLA_2$ and $cPLA_2$ inhibitors or antagonists have been reported to inhibit multiple membrane trafficking pathways in endocytosis such as the recycling of transferrin receptors and low-density lipoprotein receptors (Doody et al., 2009; Mayorga et al., 1993) and phagocytic function (De et al., 2003; Girotti et al., 2004). Since iPLA2 and cPLA2 have been shown to be involved in endocystosis as well as having a decreased activity in AD brains (Ross, Moszczynska, Erlich, & Kish, 1998; Sun et al., 2004; Talbot et al., 2000) it is logical to conclude that PLA2s may play an integral role in Aβ clearance and therefore AD pathogenesis.

1.5 Hypothesis

$cPLA_2$ and $iPLA_2$ play a pivotal role in the uptake of Aβ by microglia to facilitate Aβ clearance and attenuate the cytotoxic effects of Aβ accumulation in AD pathogenesis.

CHAPTER 2 $A\beta_{1-42}$ CHARACTERIZATION

2.1 Method

2.1.1 Materials

Human $A\beta_{1-42}$ was purchased from AnaSpec (Fremont, CA). HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) and DMSO (Dimethyl sulfoxide) were from Sigma-Aldrich (St. Louis, MO). Ham's F-12 without phenol red was from Crystalgen (Commack, NY). DMEM without phenol red (Dulbecco's Modified Eagle's Medium) DPBS without Calcium and Magnesium (Dulbecco's phosphate-buffered saline) were from Life Technologies (Grand Island, NY). Native sample buffer, 10X Tris/Glycine buffer, 10X TBS (Tris-buffered saline) and 0.45 µm nitrocellulose membrane were from Bio-Rad (Hercules, CA). 6E10 ($A\beta_{1-16}$ monoclonal antibody, mouse anti-human) was from BioLegend (Dedham, MA). Anti-mouse IgG, HRP (horseradish peroxidase)-linked antibody was from Cell Signaling Technology (Danvers, MA). SuperSignal West Pico chemiluminescent substrate was from Thermo Scientific (Rockford, IL).

2.1.2 Preparation of $A\beta_{1-42}$ oligomers

Oligomeric $A\beta_{1-42}$ was prepared according to the protocol described (Dahlgren et al., 2002). 1 mg Lyophilized $A\beta_{1-42}$ was first dissolved in HFIP to a concentration of 1 mM. The solution remained at room temperature for 1 hour. The clear

solution was aliquoted and speed vacuumed for 1 hour to evaporate HFIP, leaving an $A\beta_{1-42}$ film in the tube. The aliquots of $A\beta_{1-42}$ were stored at -20 °C.

To prepare $A\beta_{1-42}$ oligomers, $A\beta_{1-42}$ film was dissolved in 2 µL DMSO, sonicated for 5 minutes and then dissolved in 98 µL Ham's F-12 or DPBS to a concentration of 100 µM. $A\beta_{1-42}$ solution was either directly used or incubated at 4 °C for 24 hours prior to use.

2.1.3 Native-PAGE of $A\beta_{1-42}$

$A\beta_{1-42}$ solution was further diluted (x3) by native sample buffer. An 18% Tris-glycine polyacrylamide gel was prepared. 2 µL and 4 µL pre-stained protein ladder and equivalent volumes (6 µL) of $A\beta_{1-42}$ samples were loaded in sequence. Electrophoresis was operated first at 80 V for 30 minutes and 200 V later for about 3 hours until samples were moved to the bottom of the gel. Proteins were then transferred to the nitrocellulose membrane. Membrane was blocked with 5% (w/v) non-fat dry milk in TBST (TBS containing 0.1% (v/v) Tween 20) for 1 hour at room temperature. Then membrane was incubated with 6E10 (1:1000) in 5% (w/v) non-fat dry milk in TBST overnight at 4 °C on the orbital shaker. Next day, membrane was washed with TBST for 3 times, 5 minutes each. Membrane was then probed by anti-mouse HRP-linked antibody (1:2000) in 5% (w/v) non-fat dry milk in TBST for 1 hour at room temperature on the orbital shaker. After washing with TBST for 3 times, 5 minutes each, membrane was incubated with chemiluminescent substrate for 5 minutes.

Membrane was then wrapped in a plastic transparent film and visualized by myECL imaging system (Thermo Scientific). Protein band intensity was analyzed by Quantity One software (Bio-Rad, Hzercules, CA)

2.2 Results

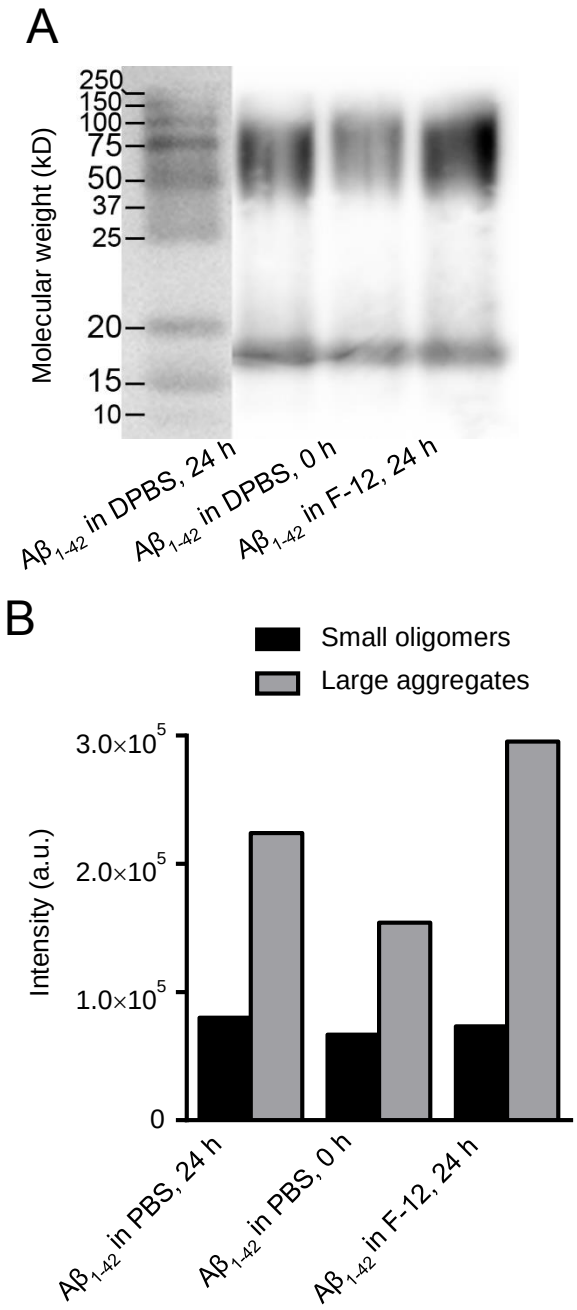


Figure 2.1. Native-PAGE for $A\beta_{1\text{-}42}$ characterization. $A\beta_{1\text{-}42}$ was incubated in either DPBS or Ham's F-12 at 4°C for 0 or 24 h. A) showed two separate bands, a band of small oligomers

around 17 kD and a band of large aggregates around 40-150 kD. B) showed the intensity of two separate bands. Values were from one independent experiment.

Native-PAGE image (Figure 2.1A) showed that all preparations had a similar $A\beta_{1-42}$ aggregation profile which contained small oligomers around 17 kD and large aggregates around 40-150 kD. The quantitative result (Figure 2.1B) showed that $A\beta_{1-42}$ incubated in either DPBS or Ham's F-12 for 0 or 24 hours had similar levels of small oligomers. $A\beta_{1-42}$ in Ham's F-12 for 24 hours had the highest level of large aggregates while $A\beta_{1-42}$ in DPBS without incubation had the lowest level of large aggregates.

2.3 Discussion

Native-PAGE showed that $A\beta_{1-42}$ aggregated into two separate bands: ~17 kD and 40-150 kD, which accorded with the reported Aβ oligomer sizes found in human brain (Kuo et al., 1996). The results suggest that $A\beta_{1-42}$ initially aggregates into tetramer sized oligomers at very early times and forms more large aggregates after 24 hours incubation in both incubating solutions. While the large aggregates increase with incubation, there presence of tetramer sized oligomers remains constant regardless of incubating solution of duration. In the subsequent experiments, 100 µM $A\beta_{1-42}$ was incubated in Ham's F-12 at 4°C for 24 hours prior to use.

Chapter 3 MICROGLIA PRETREATMENT

3.1 Method

3.1.1 Materials

The immortalized murine microglia cell line, BV-2 cells, were a generous gift from Dr. Gary A. Weisman and Dr. Grace Y. Sun (Department of Biochemistry, University of Missouri-Columbia). DMEM, heat-inactivated FBS (fetal bovine serum), PS (penicillin/streptomycin), DPBS without Calcium and Magnesium were from Life Technologies (Grand Island, NY). $A\beta_{1-42}$ oligomers were prepared as Chapter 2 described. For experiments, $A\beta_{1-42}$ solution was further diluted by phenol red-free DMEM to a final concentration of 1 μM. LPS (Lipopolysaccharides), IFNγ (Interferon-γ), MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), DMSO and monoclonal anti-β-Actin−peroxidase antibody were from Sigma-Aldrich (St. Louis, MO). RIPA buffer (Radio Immunoprecipitation Assay) was from Abcam (Cambridge, MA). 2X Laemmli sample buffer, 10X Tris/Glycine buffer, 10X Tris/Glycine/SDS buffer, 10X TBS and 0.45 μm nitrocellulose membrane were from Bio-Rad (Hercules, CA). β-ME (2-Mercaptoethanol), western blot stripping buffer were from Thermo Scientific (Rockford, IL). BSA (Bovine serum albumin) was from Fisher Scientific (Pittsburgh, PA). Protease/phosphatase inhibitor cocktail, p-$cPLA_2$ (Ser505) antibody (phospho-cytosolic phospholipase A_2), $cPLA_2$ antibody (cytosolic phospholipase A_2), anti-rabbit IgG, HRP-linked antibody were from Cell Signaling

Technology (Danvers, MA). Methyl Arachidonyl Fluorophosphonate (MAFP) and Bromoenol Lactone (BEL) were from Santa Cruz Biotechnology (Dallas, TX).

3.1.2 Cell culture

BV-2 cells were seeded and maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% PS (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified incubator at 37°C, 5% CO_2. Culture medium was replaced every 48 hours. BV-2 cells were detached from culture flasks by cell scraper and subcultured into a new flask when 80%-90% confluent. BV-2 cells were used between 15-25 passages in accordance with the supplier's recommendation.

3.1.3 Cell viability assay

Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. BV-2 cells were cultured in 96-well plate at a density of 1×10^5cells/mL overnight in DMEM + 10% heat-inactivated FBS + 1% PS. Next day, culture medium was replaced with serum-free DMEM for 6 hours. Cells were then treated with 1 μg/mL LPS and 10 ng/mL IFNγ, or 10 μM MAFP, or 2 μM BEL for 1 hour. Both MAFP and BEL were dissolved in DMSO. During treatment, dilution times of MAFP and BEL were at least 1000 times to make sure DMSO was less than 0.01% in the medium. After treatment, cells were incubated with 0.5 mg/mL MTT in DMEM for 4 hours at 37 °C. The medium was removed and the formazan was dissolved in DMSO for 10 minutes with shaking. The absorbance was read by microplate reader at 570 nm.

3.1.4 Western blot of $cPLA_2$ and p-$cPLA_2$

BV-2 cells were cultured in 35mm dishes at a density of 1×10^5 cells/mL overnight in DMEM + 10% heat-inactivated FBS + 1% PS. Next day, cells were about 70%-80% confluence. Culture medium was replaced with serum-free DMEM for 6 hours before treatment. Cells were stimulated by 1 μM $A\beta_{1-42}$ for 1 hours. Or cells were stimulated by 1 μg/mL, 5 μg/mL LPS and 10 ng/mL IFNγ for different times. After treatment, culture medium was removed. Cells were washed twice with cold DPBS and then lysed with 300 μL cold RIPA buffer supplemented with 1% protease/phosphatase inhibitor cocktail for 15 minutes. Cell lysates were collected into 1.5 mL Eppendorf tubes by cell scraper. Cell lysates were then centrifuged at 13,000 rpm for 10 minutes and supernatants were collected.

Cell samples were diluted 1:1 by laemmli sample buffer supplemented with 5% β-ME and boiled at 100 °C for 5 minutes. 10% SDS-polyacrylamide gels were prepared. 2 μL and 4 μL pre-stained protein ladder and equivalent volumes (30 μL) of samples were loaded in sequence. Electrophoresis was operated first at 80 V for 30 minutes and 120 V later for about 1.5 hours until samples were moved to the bottom of the gel. Proteins were then transferred to the nitrocellulose membrane. Based on the pre-stained protein ladder, membrane was cut into two bands: 25-50 kD and 75-150 kD. Membrane 25-50 kD was blocked with 5% (w/v) non-fat dry milk in TBST for 1 hour at room temperature and incubated in anti-β-Actin−peroxidase antibody (1:50,000) in 5% (w/v) non-fat

dry milk in TBST overnight at 4 °C on the orbital shaker. Membrane 75-150 kD was blocked with 5% BSA in TBST for 1 hour at room temperature and then incubated with p-cPLA$_2$ antibody in 5% BSA in TBST overnight at 4 °C on the orbital shaker. Next day, membranes were washed with TBST for 3 times, 5 minutes each. Membrane 75-150 kD was incubated with anti-rabbit IgG, HRP-linked antibody (1:2000) in 5% BSA in TBST at room temperature for 1 hour. Membrane 75-150 kD was again washed with TBST for 3 times, 5 minutes each. Membrane 25-50 kD and membrane 75-150 kD were incubated with chemiluminescent substrate for 5 minutes. After that, membranes were wrapped in a plastic transparent film and visualized by myECL imaging system (Thermo Scientific). Membrane 75-150 kD was washed with TBST and then incubated in stripping buffer at room temperature for 20 minutes. Membrane 75-150 kD was washed with TBST 6 times, 5 minutes each. Similarly, cPLA$_2$ band was visualized on the membrane 75-150 kD. Protein band intensity were analyzed by Quantity One software (Bio-Rad, Hercules, CA).

3.1.4 Statistical analysis

Data were presented as mean ± SD from at least three independent experiments. Statistical comparison between two groups was made with Student's t- test; Statistical comparison among three or more groups were carried out with one-way ANOVA followed by Tukey's post-hoc comparison in GraphPad Prism 6.01. Value of $p<0.05$ was considered statistically significant.

3.2 Results

3.2.1 $A\beta_{1-42}$-induced $cPLA_2$ activation in BV-2 cells

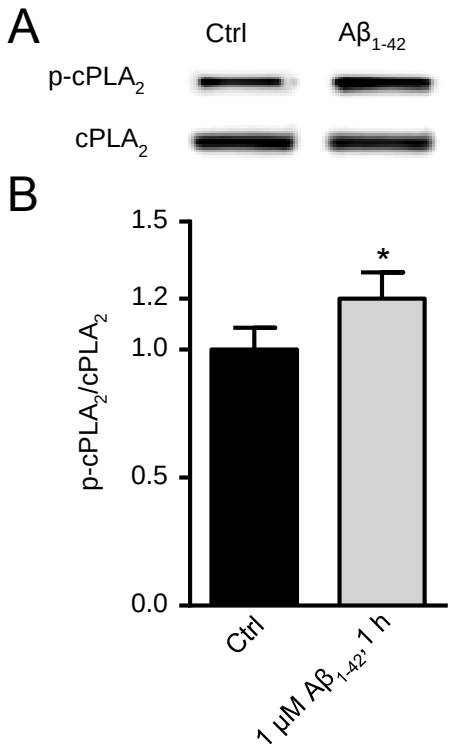


Figure 3.1. Western blot analysis of Aβ-induced $cPLA_2$ activation in BV-2 cells. BV-2 cells were treated with or without 1 μM $A\beta_{1-42}$ for 1 h and the cell lysates were run through a 10% SDS-PAGE gel. A) Western blot analysis was performed to determine the quantities of phosphorylated $cPLA_2$ (p-$cPLA_2$) and total $cPLA_2$. B) The ratio of p-$cPLA_2$/$cPLA_2$ is represented as the fraction percentage of the Ctrl group. Data is shown as mean ± SD from four independent experiments with statistical comparisons carried out with Student t-test. (*p< 0.05, compared with Ctrl group)

Bv2 cells were treated with 1 μM $A\beta_{1-42}$ for 1 hour which revealed that there was a 20% increase in $cPLA_2$ phosphorylation (p-$cPLA_2$) when compared to the control group according to Western blot analysis (Figure 3.1). Similarly, when the cells were incubated with 10 μM IFNγ and 1 or 5 μM LPS for various times there was a 50-80% increase in p-$cPLA_2$. As shown in Figure 3.2A, all groups treated

with LPS and IFNγ showed an increased level of p-cPLA$_{2,}$ but no change in total cPLA$_2$. The quantitative results show that there is no significant difference between 1 and 5 μg/mL LPS groups at the same time (Figure 3.2B).

3.2.2 Cell viability test on BV-2 cells

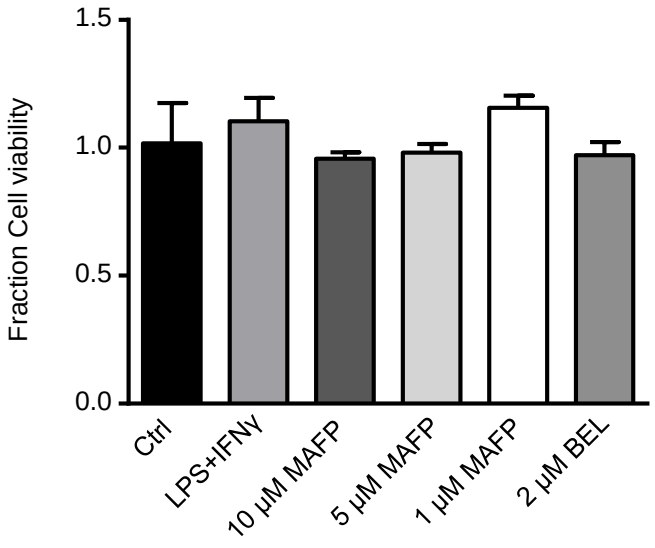


Figure 3.2. BV-2 cell viability assays. BV-2 cells were with LPS (1 μg/mL) + IFNγ (10 ng/mL), MAFP (10 μM, 5 μM, 1 μM) or BEL (2 μM) for 1 h. The data is presented as the fraction cell viability of the Ctrl group with the mean ± SD from six independent experiments represented. Statistical comparisons were carried out with a Student t-test. (**p< 0.01, ***p< 0.001 compared with Ctrl group)

MAFP inhibits $cPLA_2$ and $iPLA_2$; BEL inhibits $iPLA_2$. MTT assay showed that with 1 hour treatment of LPS (1 μg/mL) + IFNγ (10 ng/mL), MAFP (10 μM, 5 μM, 1 μM) or BEL (2 μM) there was no significant difference in cell viability when comparted to the control group.

3.2.3 LPS & IFNγ-induced $cPLA_2$ activation in BV-2 cells

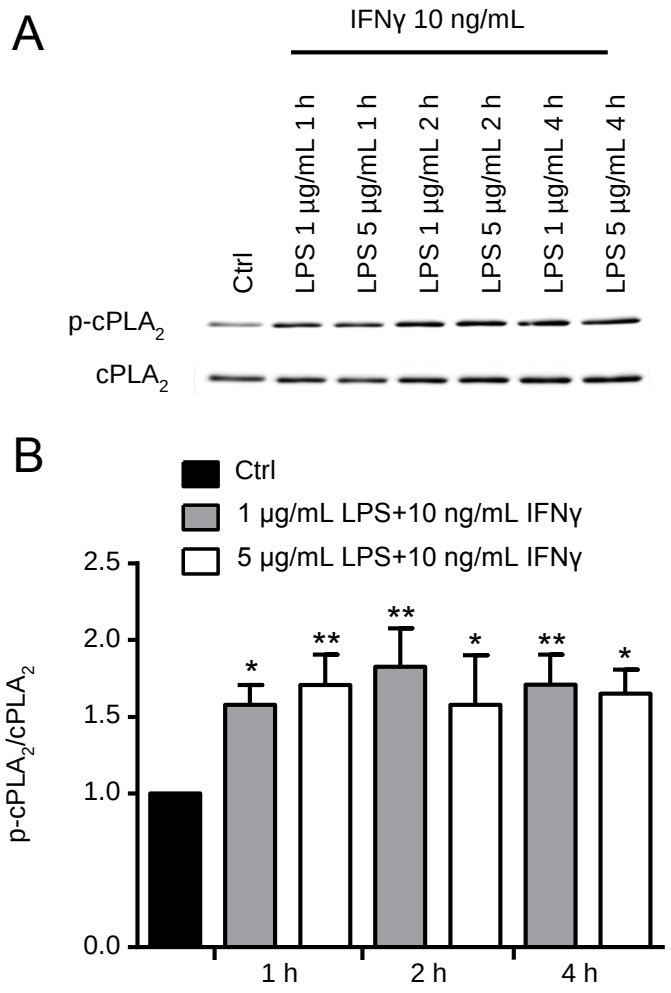


Figure 3.3. Western blot of LPS & IFNγ -induced $cPLA_2$ activation in BV-2 cells. BV-2 cells were treated with 1 or 5 μg/mL LPS at various times and 10 ng/mL IFNγ. A) A representative Western blot of the p-$cPLA_2$ and total $cPLA_2$ expression levels with the various treatment groups. B) The ratio of p-$cPLA_2$/$cPLA_2$ was represented as the fraction percentage of the Ctrl group. Data is shown as mean ± SD from three independent experiments with statistical comparisons carried out with one-way ANOVA followed by Tukey's post-hoc test. (*$p< 0.05$, **$p< 0.01$ compared with Ctrl group)

3.3 Discussion

BV-2 cells shared similar properties with primary microglia and was considered to be a suitable alternative model (Henn et al., 2009; Stansley, Post, & Hensley, 2012). $cPLA_2$ contains phosphorylation site at Ser 505 that up-regulates the enzymatic activity. Previous research has shown that oligomeric $A\beta_{1-42}$ activates $cPLA_2$ in neurons and astrocytes (Kriem et al., 2005; Zhu et al., 2006). Here western blot analysis shows that $A\beta_{1-42}$ increases $cPLA_2$ activation by approximately 20%.

Several common stimulators such as LPS, IFNγ and TNFα are known to induce $cPLA_2$-related signaling pathways through mitogen-activated protein kinases (MAPKs) (Clark, Schievella, Nalefski, & Lin, 1995; Leslie, 1997). LPS is a major component of the outer membrane of Gram-negative bacteria. IFNγ is a cytokine produced by lymphocytes and a natural killer cells. They both have been reported to induce activation of $cPLA_2$ in various cell types (Leslie, 1997; Qi & Shelhamer, 2005). Furthermore, IFNγ was reported to amplify LPS-induced signaling pathway by promoting MyD88-dependent and independent pathways which further activates the downstream signaling machinery, including MAPK pathways (Schroder, Hertzog, Ravasi, & Hume, 2004). Thus, LPS and IFNγ were used together to stimulate BV-2 cells. Western blot analysis showed a constant p-$cPLA_2$ elevation after LPS&IFNγ stimulation, 50-80%, regardless of treatment time or LPS concentration. BV-2 cells were treated with 1 μg/mL LPS and 10 ng/mL IFNγ for 1 hour as a standard protocol for the subsequent experiments.

CHAPTER 4 $cPLA_2$ IS REQUIRED FOR $A\beta_{1-42}$ UPTAKE IN BV-2 CELLS

4.1 Method

4.1.1 Materials

The immortalized murine microglia cell line, BV-2 cells, were a generous gift from Dr. Gary A. Weisman and Dr. Grace Y. Sun (Department of Biochemistry, University of Missouri-Columbia). DMEM, heat-inactivated FBS, PS, DPBS without Calcium and Magnesium, commercial $A\beta_{1-42}$ ELISA kit, antifade mountant with DAPI were from Life Technologies (Grand Island, NY). $A\beta_{1-42}$ oligomers were prepared as described in Chapter 2. For experiments, $A\beta_{1-42}$ solution was further diluted in phenol red-free DMEM to a final concentration of 1 μM. Protease inhibitor cocktail, LPS, IFNγ, paraformaldehyde (PFA), Triton X-100, goat serum were from Sigma-Aldrich (St. Louis, MO). RIPA buffer was from Abcam (Cambridge, MA). BCA kit was from Thermo Scientific (Rockford, IL). MAFP and BEL were from Santa Cruz Biotechnology (Dallas, TX). Poly-L-lysine pre-coated coverslips were from Neuvitro (Vancouver, WA). Alexa Flour 488-6E10 was from BioLegend (Dedham, MA).

4.1.2 $A\beta_{1-42}$ uptake and quantification

BV-2 cells were cultured in 35mm dishes at a density of 1×10^5cells/mL overnight in DMEM + 10% heat-inactivated FBS + 1% PS. Next day, cells were about 70%-80% confluence. Culture medium was replaced with serum-free DMEM for 6

hours before treatment. Cells were pretreated with 10 μM, 5 μM, 1 μM MAFP or 2 μM BEL for 30 minutes and remained in the medium during whole treatment. Then cells were stimulated by 1 μg/mL LPS and 10 ng/mL IFNγ for 1 hour, followed by incubating with 1 μM $A\beta_{1-42}$ for 15, 30, 45, 60 minutes. Both MAFP and BEL were dissolved in DMSO. During treatment, dilution times of MAFP and BEL were at least 1000 times to make sure DMSO was less than 0.01% in the medium. After treatment, cells were washed twice with cold DPBS and then lysed with 300 μL cold RIPA buffer supplemented with 1% protease inhibitor cocktail for 15 minutes. Cell lysates were collected into 1.5 mL Eppendorf tubes by cell scraper. Cell lysates were then centrifuged at 130,000 rpm for 10 minutes and supernatants were collected.

Total protein concentrations in supernatants are determined by BCA kit. According to the manufacture's protocol, a set of diluted standards was prepared in RIPA buffer. Same volume of diluted standards and unknown samples were added into 96-well plate in duplicate. Then working reagents were added into each well. 96-well plate was incubated at 37 °C for 30 minutes. The absorbance was measured at 562 nm on a microplate reader (BioTek Synergy H1). The absorbance related to protein concentration was plotted as standard curve. Based on the standard curve, the total protein concentration of samples could be calculated. Equivalent amounts of total protein for each sample were adjusted.

ELISA assay of $A\beta_{1-42}$ was performed according to the manufacturer's instructions. Human $A\beta_{1-42}$ diluted standards and samples were added into 96-

well plate. Detection antibody was then added into each well and the plate was sealed and incubated at 4 °C overnight. Next day, the solution was aspirated from each well. Excess antibody was removed by wash buffer. Anti-Rabbit IgG HRP was added into each well as secondary antibody and the plate was incubated at room temperature for 30 minutes. Excess antibody was removed by wash buffer. Stabilized Chromogen solution was added into each well and the plate was incubated at room temperature in dark. After 15 minutes, stop solution was added. The absorbance was measured at 450 nm by microplate reader. The absorbance related to $A\beta_{1-42}$ concentration was plotted as standard curve. Based on the standard curve, $A\beta_{1-42}$ concentration of samples could be calculated.

4.1.3 Immunofluorescence imaging

BV-2 cells were grown on poly-L-lysine pre-coated coverslips in 12-well plate at a density of 8×10^4 cells/mL overnight in culture medium. Next day, cells were about 40%- 50% confluence. Cells were treated as previously described in $A\beta_{1-42}$ uptake and quantification except for only using 10 μM MAFP. After treatment, cells were washed twice with cold DPBS and fixed with 4% PFA (w/v) in DPBS for 15 minutes. After washing 3 times with DPBS, cells were permeabilized with 0.3% Triton X-100 (v/v) in DPBS for 10 minutes and again washed 3 times with DPBS. Then cells was blocked with 5% goat serum (v/v) in DPBS for 1 hour at room temperature and incubated with Alexa Flour 488-6E10 (1:200) in 1%BSA (w/v) in DPBS at 4 °C overnight in dark. Next day, cells were washed 3 times with

DPBS and allowed to dry. Then coverslips were mounted onto slides with antifade mountant with DAPI and kept at room temperature in dark for 24 hours before imaging. Fluorescent images were acquired by a Nikon TE-2000U fluorescence microscope with an oil immersion, 60X objective, using a cooled CCD camera. At least 12 images were acquired from each slide. Quantitative results were analysis by MetaVue software and data were normalized by cell number.

4.1.4 Statistical Analysis

Data were presented as mean ± SD from at least three independent experiments. Statistical comparison between two groups was made with Student's t- test; Statistical comparison were carried out with one-way ANOVA followed by Tukey's post-hoc comparison in GraphPad Prism 6.01. Value of $p<0.05$ was considered statistically significant.

4.2 Results

4.2.1 $A\beta_{1-42}$ uptake by BV-2 cells (ELISA)

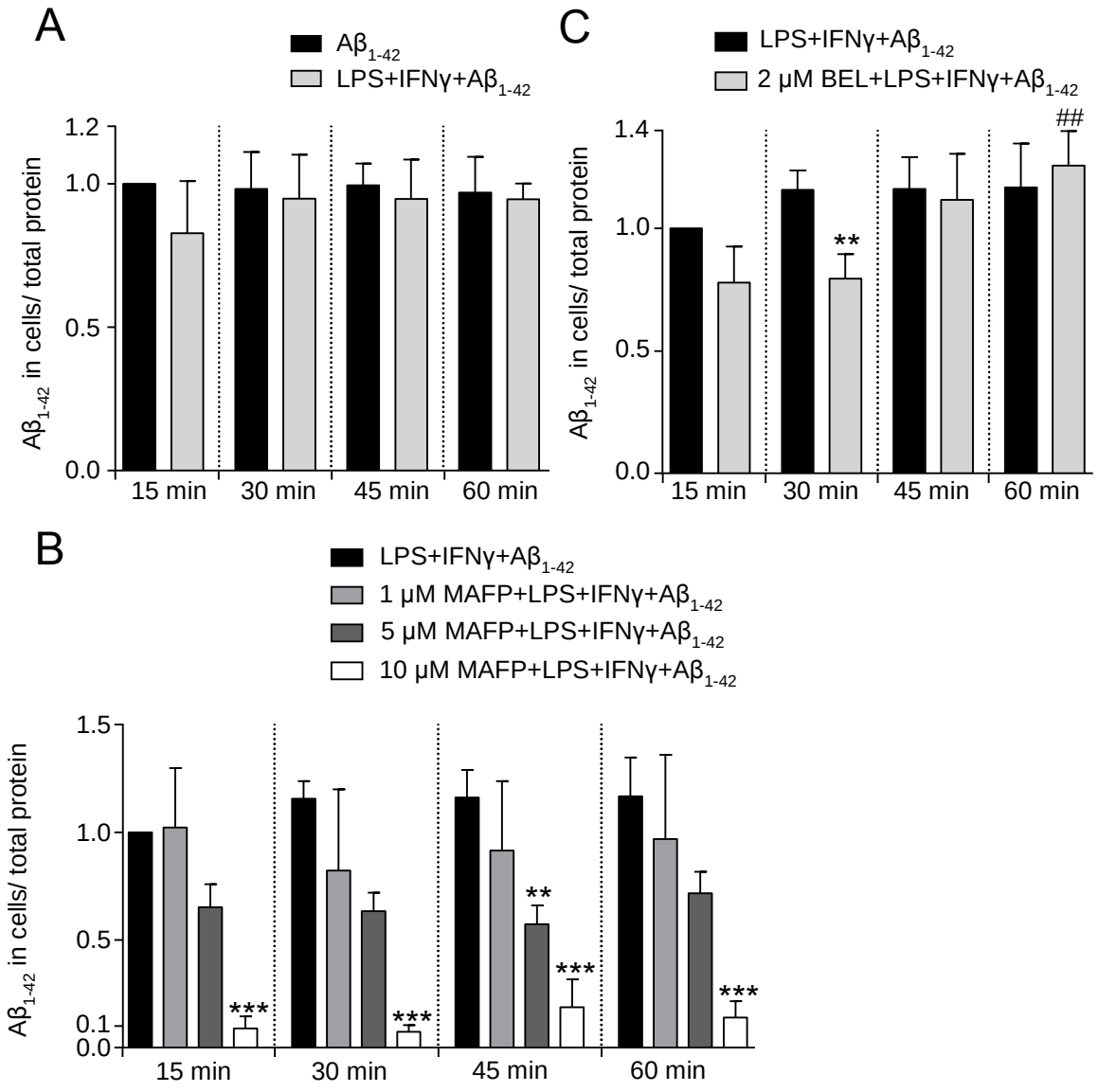


Figure 4.1. Time course study of $A\beta_{1-42}$ uptake by BV-2 cells. ELISA assays were performed to quantify the presence of $A\beta_{1-42}$ in the cell lysates. BCA assays were performed to assess the total protein in the cell lysates. Data is presented as the ratio of $A\beta_{1-42}$ / total protein and normalized by their respective controls at 15 min. The data is represented as the mean ± SD from three independent experiments and statistical analysis performed with Student t-tests. A) BV-2 cells

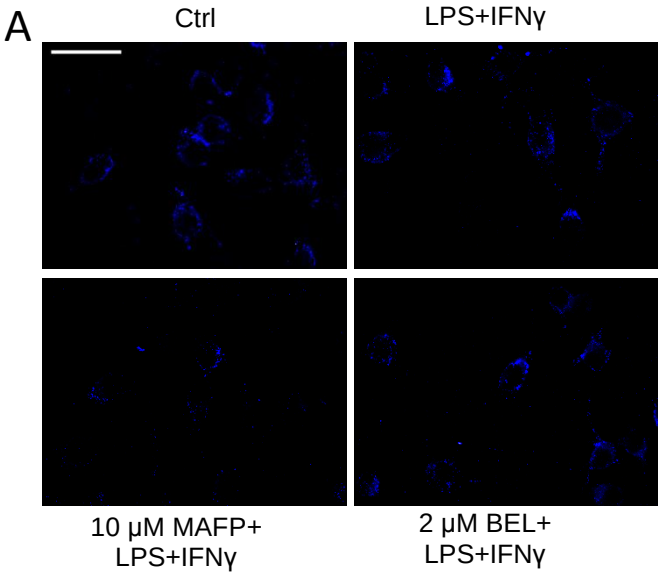
were treated with or without LPS (1 μg/mL) + IFNγ (10 ng/mL) for 1 h followed by 1 μM $A\beta_{1-42}$ treatment for 15, 30, 45 and 60 min. No significant difference was observed between these two groups at any one time. B) BV-2 cells were treated with or without MAFP (1 μM, 5 μM, 10 μM) for 30 min followed by a 1 h treatment of LPS (1 μg/mL) + IFNγ (10 ng/mL). Then, $A\beta_{1-42}$ (1 μM) was added to the media and allowed to incubate for 15, 30, 45 and 60 min. (*p<0.05, **p< 0.01, ***p<0.001 compared with LPS+IFNγ+$A\beta_{1-42}$ at the corresponding time) C) BV-2 cells were treated with or without BEL (2 μM) for 30 min followed by a 1 h treatment of LPS (1 μg/mL) + IFNγ (10 ng/mL). Then, $A\beta_{1-42}$ (1 μM) was added to the media and allowed to incubate for 15, 30, 45 and 60 min. (**p< 0.01 compared with LPS+IFNγ+$A\beta_{1-42}$ at the corresponding time, ##p<0.01 compared with BEL+LPS+IFNγ+$A\beta_{1-42}$ at 15 and 30 min)

$A\beta_{1-42}$ uptake in normal BV-2 cells reached a plateau before15 minutes with no discernable change over 60 minutes. Upon increased $cPLA_2$ activation with LPS and IFNγ $A\beta_{1-42}$ uptake was slightly lower at 15 minutes and reached a plateau at 30 minutes with no significant difference between either groups at any time (Figure 4.2A). The data shows that increased $cPLA_2$ activation did not affect $A\beta_{1-42}$ uptake. Without $A\beta_{1-42}$ incubation, no $A\beta_{1-42}$ was detected in BV-2 cells (data not shown).

When BEL was introduced into the media there appears to be a gradual increase of $A\beta_{1-42}$ uptake as incubation time increases from 15 to 60 minutes (Figure 4.2B). $A\beta_{1-42}$ uptake at 60 minutes was significantly higher than 15 minutes and 30 minutes. When MAFP is introduced into the media at various concentrations (1, 5 and 10 μM) there is a very clear decrease in $A\beta_{1-42}$ uptake by LPS&IFNγ activated BV-2 cells (Figure 4.2C). In fact, $A\beta_{1-42}$ uptake appears to be dose

dependent with 10 μM MAFP having the least amount of $A\beta_{1-42}$ within the cells. Still, there were no difference of $A\beta_{1-42}$ in the cell lysates as incubation time increased from 15 to 60 minutes.

4.2.2 $A\beta_{1-42}$ uptake by BV-2 cells (Immunofluorescence imaging)



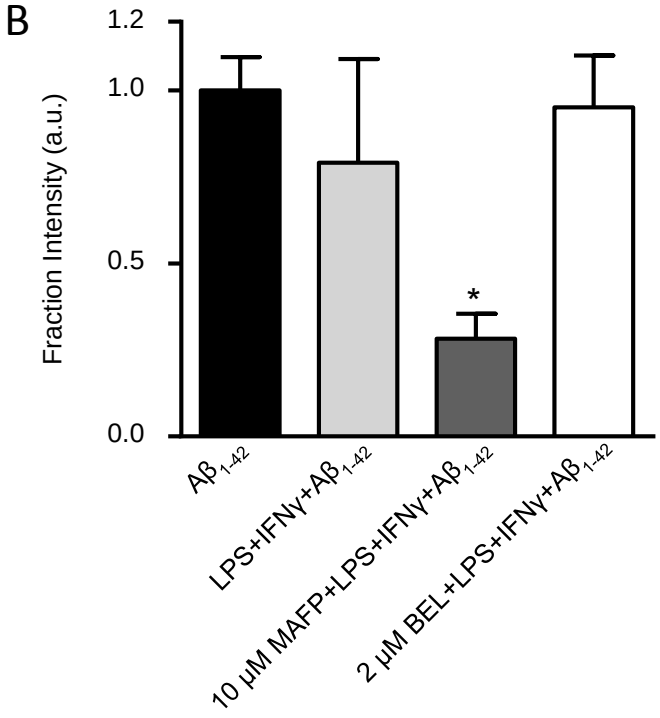


Figure 4.2. Immunofluorescence imaging analysis of $A\beta_{1-42}$ uptake. BV-2 cells were treated with MAFP (10 μM) or BEL (2 μM) for 30 min followed by a 1 h LPS (1 μg/mL) + IFNγ (10 ng/mL) treatment for all groups except the Ctrl. Then, $A\beta_{1-42}$ was added to the media and allowed to incubate for 15 min. A) Representative images of each treatment group after incubation with a fluorescent anti-Aβ antibody (Alexa Fluor 488-6E10). Scale bar: 35 μm B) Data was analyzed and quantified as the overall fluorescent intensity and normalized by the Ctrl group ($A\beta_{1-42}$ only treatment). Values are shown as the fraction intensity of the $A\beta_{1-42}$ group and represented as the mean ± SD from three independent experiments (at least 12 images/experiment). Statistical comparisons were carried out with one-way ANOVA followed by Tukey's post-hoc test (*$p < 0.05$ compared with LPS+ IFNγ+$A\beta_{1-42}$ group).

To confirm the ELISA results, immunofluorescence was performed on BV-2 cells with the same treatment groups after 15 minutes of incubation. Figure 4.3 reveals that the immunofluorescent results are similar to the ELISA in Figure 4.2. Activation of $cPLA_2$ by LPS and IFNγ did not affect $A\beta_{1-42}$ uptake significantly and BEL does not appear to affect $A\beta_{1-42}$ uptake. However, consistent with the aforementioned ELISA results, MAFP significantly reduces $A\beta_{1-42}$ uptake by approximately 70% within 15 minutes of incubation (Figure 4.3).

4.3 Discussion

Our result showed $A\beta_{1-42}$ uptake by BV-2 cells occurred very fast (<15 minutes) and did not change over the remaining hour, implying that under continuous exposure of $A\beta_{1-42}$, microglia cannot change its rate or capacity of $A\beta_{1-42}$ uptake. It appears that the phagocytosis of $A\beta_{1-42}$ occurs too quickly to detect with these experimental methods. Microglia were reported to clear an apoptotic cell in 25-90

minutes (Sierra, Abiega, Shahraz, & Neumann, 2013), so the phagocytosis of smaller particles such as oligomer $A\beta_{1-42}$ should occur much quicker. The internalized $A\beta_{1-42}$ measured was the full length peptide due to the sandwich ELISA kit, with the actual internalized $A\beta_{1-42}$ measuring 200-400 pg/μg total protein. Due to the lack of change of internalized $A\beta_{1-42}$ between 15 to 60 minutes it is likely that this is maximum capacity of $A\beta_{1-42}$ within microglia.

Our result suggested that $cPLA_2$ activation prior to $A\beta_{1-42}$ exposure does not improve or impair $A\beta_{1-42}$ uptake by microglia, Evidences had showed that LPS inhibited fibrillar $A\beta_{1-42}$ phagocytosis by primary microglia and BV-2 cells (Koenigsknecht-Talboo & Landreth, 2005), since it is unlikely that ELISA is able to detect fibrils due to their high molecular weight, one possibility is that fibrillar $A\beta_{1-42}$ and oligomeric $A\beta_{1-42}$ have different ways of phagocytosis by microglia.

To explore whether inhibiting $cPLA_2$ would have influence on $A\beta_{1-42}$ uptake, a pharmaceutical inhibitor MAFP was used to inhibit $cPLA_2$ activity. There are several commercially available $cPLA_2$ inhibitors such as MAFP, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and pyrrophenone. MAFP is a selective, irreversible inhibitor of $cPLA_2$ and $iPLA_2$, of which IC_{50} reported is 5 μM and 0.5 μM respectively (IC_{50} is the concentration of the inhibitor that inhibits 50% activity of the substrate); $AACOCF_3$ is a reversible and slow-binding inhibitor of $cPLA_2$ and $iPLA_2$ (Choudhury, McKay, Flower, & Croxtall, 2000); Pyrrophenone is a specific, reversible inhibitor of $cPLA_2$. MAFP was chosen due to its irreversible property. Different concentrations of MAFP (1 μM, 5 μM and 10 μM) were chosen to treat BV-2 cells before $A\beta_{1-42}$ uptake. During the whole treatment, MAFP

remained in the medium since $A\beta_{1-42}$ could trigger $cPLA_2$ activation as well. Our result showed that MAFP could inhibit $A\beta_{1-42}$ uptake in a dose-dependent manner. With 10 μM MAFP, microglia had nearly no phagocytosis of $A\beta_{1-42}$.

To exclude the possibility that MAFP non-specifically inhibits $iPLA_2$ activity, another pharmaceutical inhibitor BEL was used to specifically inhibit $iPLA_2$ activity, whose IC_{50} is 60 nM. We found BEL slows down $A\beta_{1-42}$ uptake within a short time, allowing us to catch the rising phase, but did not affect uptake later on. We speculate that inhibiting $iPLA_2$ had an inhibitory effect on $A\beta_{1-42}$ uptake at first, but later this effect was compensated.

The ELISA assay detections the full $A\beta_{1-42}$ peptide due to its sandwich property, while the immunofluorescent images use an antibody that specifically binds to $A\beta_{1-16}$ (6E10). Nonetheless, the results showed consistent trends where a 10 μM MAFP treatment greatly reduced $A\beta_{1-42}$ internalization. Interestingly, there was slightly more $A\beta_{1-42}$ internalization with immunofluorescence imaging than in ELISA with the MAFP treatment group. This is likely because immunofluorescence is slightly less accurate and/or 6E10 may be binding to Aβ fragments.

In case $A\beta_{1-42}$ was not internalized by microglia, but instead bound to the membrane, immunofluorescence imaging was conducted where the cell membrane was not permeabilized prior to treatment with the fluorescent antibody. $A\beta_{1-42}$ fluorescent signal was too low to detect which confirmed that most $A\beta_{1-42}$ was internalized (Data not shown).

CHAPTER 5 $cPLA_2$ IS NOT REQUIRED FOR $A\beta_{1-42}$ DEGRADATION IN BV-2 CELLS

5.1 Method

5.1.1 Materials

The immortalized murine microglia cell line, BV-2 cells, were a generous gift from Dr. Gary A. Weisman and Dr. Grace Y. Sun (Department of Biochemistry, University of Missouri-Columbia). DMEM, heat-inactivated FBS, PS, DPBS without Calcium and Magnesium, $A\beta_{1-42}$, commercial ELISA kit were from Life Technologies (Grand Island, NY). $A\beta_{1-42}$ oligomers were prepared as Chapter 2 described. For experiments, $A\beta_{1-42}$ solution was further diluted by phenol red-free DMEM to a final concentration of 1 μM. Protease inhibitor cocktail, LPS, IFNγ and NH_4Cl were from Sigma-Aldrich (St. Louis, MO). RIPA buffer was from Abcam (Cambridge, MA). BCA kit was from Thermo Scientific (Rockford, IL). MAFP and BEL were from Santa Cruz Biotechnology (Dallas, TX).

5.1.2 Cell viability assay

Cell viability was measured by MTT assay. BV-2 cells were cultured in 96-well plate at a density of 1×10^5cells/mL overnight in DMEM + 10% heat-inactivated FBS + 1% PS. Next day, culture medium was replaced with serum-free DMEM for 6 h. Cells were then treated with NH_4Cl (10 mM, 20 mM) for 1 h. After treatment, cells were incubated with 0.5 mg/mL MTT in DMEM for 4 h at 37 °C.

The medium was removed and the formazan was dissolved in DMSO for 10 minutes with shaking. The absorbance was read by microplate reader (BioTek Synergy H1) at 570 nm.

5.1.3 $A\beta_{1-42}$ depletion and quantification

BV-2 cells were cultured in 35mm dishes at a density of 1×10^5cells/mL overnight in DMEM + 10% heat-inactivated FBS + 1% PS. Next day, cells were about 70%-80% confluence. Culture medium was replaced with serum-free DMEM for 6 h before treatment, followed by incubating with serum-free DMEM, or with LPS (1 μg/mL) + IFNγ (10 ng/mL) for 1 h or with NH_4Cl (20 mM; Sigma-Aldrich) for 0.5 h. Then $A\beta_{1-42}$ (1 μM) was loaded on cells for 15 minutes and replaced by serum-free DMEM. After washing twice with serum-free DMEM, cells were collected or further incubated for 5, 15, 30 and 60 minutes in the presence of LPS (1 μg/mL) + IFNγ (10 ng/mL), or MAFP (10 μM), or BEL (2 μM) or NH_4Cl (20 mM) before lysed. NH_4Cl treatment was served as a non-processing control, thus cells were immediately lysed or further incubated for 60 minutes before lysed. $A\beta_{1-42}$ was measured as previously described in $A\beta_{1-42}$ uptake and quantification in Chapter 4.

5.1.4 Statistical analysis

Data were presented as mean ± SD from at least three independent experiments. Statistical comparison between two groups was made with Student's t- test; Statistical comparison were carried out with one-way ANOVA

followed by Tukey's post-hoc comparison in GraphPad Prism 6.01. Value of $p<0.05$ was considered statistically significant.

5.2 Results

5.2.1 Cell viability test on BV-2 cells

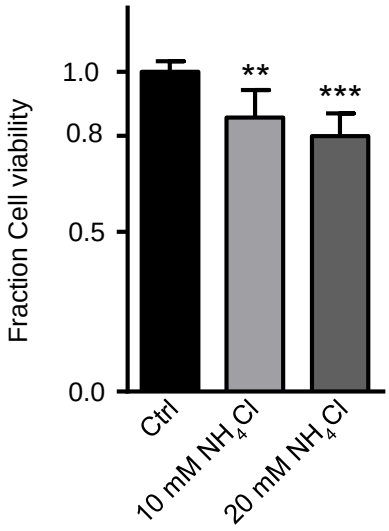


Figure 5.1. BV-2 cell viability assays. BV-2 cells were treated with NH_4Cl (10 mM, 20 mM) for 1 h. MTT assays were performed to assess cell viability after treatment. The data is presented as the fraction cell viability of the Ctrl group with the mean ± SD from six independent experiments represented. Statistical comparisons were carried out with a Student t-test. (**p< 0.01, ***p< 0.001 compared with Ctrl group)

NH_4Cl treatment was served as a control, because NH_4Cl is a lysosomotropic agent that accumulates in lysosome and increase lysosomal PH, thus inhibiting lysosomal function such as inhibiting endosome-lysosome fusion and inactivating some proteolytic enzymes resident in lysosomes (Hart & Young, 1991; Tanaka, Li, Fogelman, & Edwards, 1986). MTT assay showed that BV-2 cell viability was significantly reduced, but remained above 80% viable after 1 hour treatment of NH_4Cl (10 mM, 20 mM) when compared to the control group.

5.2.2 $A\beta_{1-42}$ depletion by BV-2 cells

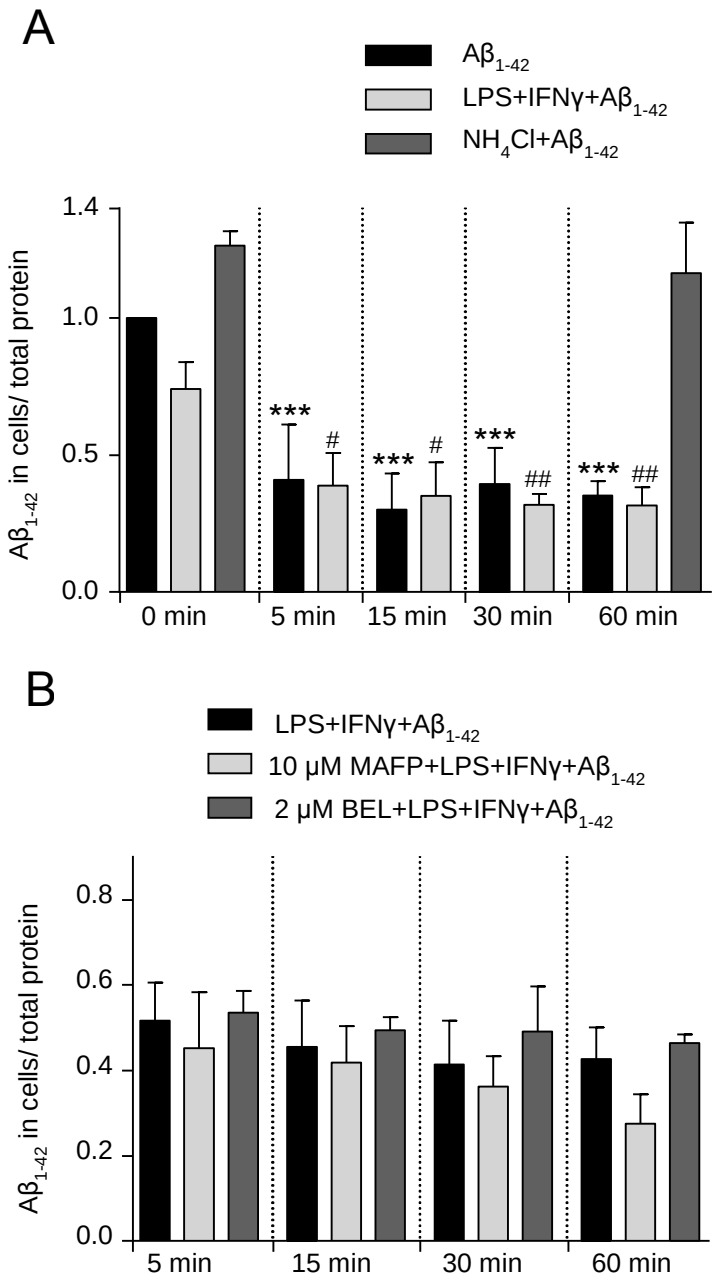


Figure 5.2. Time course of $A\beta_{1-42}$ processing by BV-2 cells. ELISA assays were performed to quantify the presence of $A\beta_{1-42}$ in the cell lysates. BCA assays were performed to assess the total protein in the cell lysates. Data is presented as the ratio of $A\beta_{1-42}$ / total protein and normalized by

their respective controls at 0 min. The data is represented as the mean ± SD from three independent experiments. A) BV-2 cells were treated with serum-free media, LPS (1 μg/mL) + IFNγ (10 ng/mL) or NH_4Cl (20 mM) for 1 h followed by $A\beta_{1-42}$ (1 μM) incubation for 15 min. Afterwards the media was replaced with the standard serum-free media and allowed to incubate for 5, 15, 30 and 60 min before lysis. In the case of the 0 min treatment groups the cells were immediately lysed. NH_4Cl inhibits lysosomal function so this treatment served as the positive control at 0 and 60 min. Statistical comparisons were carried out for every treatment group as well as within each time point using one-way ANOVA followed by Tukey's post-hoc comparison. (***$p < 0.001$ compared with 0 min $A\beta_{1-42}$ group; #$p<0.05$, ##$p < 0.01$ compared with LPS+IFNγ+$A\beta_{1-42}$, 0 min group) No significant difference existed between $A\beta_{1-42}$ group and LPS+IFNγ+$A\beta_{1-42}$ group within each time point. No significant difference existed between NH_4Cl 0 min and 60 min groups. B) BV-2 cells were treated with LPS (1 μg/mL) + IFNγ (10 ng/mL) for 1 h followed by 15 min incubation with $A\beta_{1-42}$ (1 μM). Afterwards, cells were treated with serum-free media, MAFP (10 μM) or BEL (2 μM) for 5, 15, 30 and 60 min before cell lysis. Values are shown as the fraction $A\beta_{1-42}$ / total protein of the LPS+IFNγ+ $A\beta_{1-42}$ 0 min group (shown in Figure 5A) with the mean ± SD from three independent experiments. Statistical comparison were carried out within each time point using one-way ANOVA followed by Tukey's post-hoc comparison. There were no significant differences observed.

In an effort to determine how PLA_2s participated in the removal or degradation of $A\beta_{1-42}$ in microglia, BV-2 cells were pretreated with $A\beta_{1-42}$ for 15 minutes (as well as the indicated treatment groups) and ELISA tests were run after $A\beta_{1-42}$ was removed from the media at various times. The results should offer insight into how PLA_2 inhibitors affected $A\beta_{1-42}$ intercellular presence with respect to time. Interestingly, there was no significant difference between normal cells and $cPLA_2$ activated cells with LPS and IFNγ. Within 5 minutes intercellular $A\beta_{1-42}$ decreased

more than 50% within the BV-2 cells and remained constant thereafter (Figure 5.2A). In the presence of NH_4Cl, a lysosome inhibitor, intercellular $A\beta_{1-42}$ was initially higher than the control group and remained greater than the control through 60 minutes (Figure 5.2A). In Figure 5.2B, $A\beta_{1-42}$ in cells under MAFP treatment showed an obvious gradual decrease from 15 minutes to 60 minutes while $A\beta_{1-42}$ in cells under LPS&IFNγ or BEL did not have an obvious downslope trend. At 60 minutes, MAFP-treatment group had a slightly lower Aβ1-42 left in cells compared to other two groups, though not significant, it is likely that inhibiting $cPLA_2$ has a little inhibition effect on $A\beta_{1-42}$ depletion. $iPLA_2$ did not affect $A\beta_{1-42}$ depletion.

5.3 Discussion

To study the role of PLA_2 in $A\beta_{1-42}$ depletion, BV-2 cells were manipulated similarly. LPS, MAFP or BEL treatment did not affect the $A\beta_{1-42}$ in cells, which indicates that $cPLA_2$ and $iPLA_2$ are not involved in the depletion of $A\beta_{1-42}$ after uptake.

Though almost 50% reduction of $A\beta_{1-42}$ was observed in cells, because of the limitations of the experiment, it is unclear whether the internalized $A\beta_{1-42}$ in microglia was degraded. It has been reported that microglia are unable to degrade oligomer $A\beta_{1-42}$ but release $A\beta_{1-42}$ rapidly after internalization instead (Chung, Brazil, Soe, & Maxfield, 1999). Recent studies also showed that after Aβ uptake, Aβ was rapidly trafficking to lysosome and degraded (Butler et al., 2011; Xiao et al., 2014). When BV-2 cells were treated with NH_4Cl, a lysosomal inhibitor, there was no decrease in intracellular $A\beta_{1-42}$, it is more likely that $A\beta_{1-42}$

was degraded in our case. After removal of Aβ for 1 hour, there was still 50% $A\beta_{1-42}$ left in cells with full length, which might be due to the maximal capacity of microglia or lack of enough time.

CHAPTER 6 SUMMARY AND FUTURE WORK

The purpose of this study was to investigate the effects of PLA_2s on $A\beta_{1-42}$ processing in microglial cells. This thesis work illustrates that $cPLA_2$ and $iPLA_2$ (to a lesser extent) are directly involved in the uptake of $A\beta_{1-42}$ by microglia. While inhibition of $cPLA_2$ drastically reduces intercellular $A\beta_{1-42}$ over long times and inhibition of $iPLA_2$ reduces the rate of $A\beta_{1-42}$ uptake at early times, neither PLA_2 participates in $A\beta_{1-42}$ depletion. Furthermore, the results suggest that $A\beta_{1-42}$ is reduced or degraded within the lysosomes of microglia.

The phagocytosis of $A\beta_{1-42}$ relates to several receptors in microglia, including Toll-like receptor 2 (TRL2), TRL4, SR-A, SR-B1etc (Block, Zecca, & Hong, 2007; Noda & Suzumura, 2012; Sierra et al., 2013). $cPLA_2$ activation was regulated by TRL4 (Qi & Shelhamer, 2005), because we lack an in-depth study in the underlying mechanism of $A\beta_{1-42}$ uptake related to PLA_2s, we could only speculate that inhibiting $cPLA_2$ might affect TRL4 function and cause the inhibition on $A\beta_{1-42}$ uptake. Future work should focus on the underlying mechanism behind the inhibitory effect of $cPLA_2$ on Aβ uptake by microglia and more detailed phagocytic behaviors of microglia. Similarly, future studies should address how $A\beta_{1-42}$ is processed within microglia and the mechanism of $A\beta_{1-42}$ depletion or degradation.

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