AMYLOID-β PEPTIDE INDUCES TEMPORAL MEMBRANE BIPHASIC CHANGES IN ASTROCYTES THROUGH CYTOSOLIC PHOSPHOLIPASE A_2

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by

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

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

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ABSTRACT

Oligomeric amyloid-β peptide (Aβ) is known to induce cytotoxic effects and damage cell functions in Alzheimer's disease. However, mechanisms underlying the effects of Aβ on cell membranes have yet to be fully elucidated. In this study, Aβ 1-42 ($Aβ_{42}$) was shown to cause a temporal biphasic change in membranes of astrocytic DITNC cells using fluorescence microscopy of Laurdan. $Aβ_{42}$ made astrocyte cell membranes become more molecularly-disordered after 30 minutes to 1 hour, transitioning to more molecularly-ordered after 3 hours. However, $Aβ_{42}$ caused artificial vesicle membranes made of rat whole brain lipid extract to become more disordered only. The trend for more molecularly-ordered membranes in astrocytes was abrogated by either an NADPH oxidase inhibitor, apocynin, or an inhibitor of cytosolic phospholipase A_2 ($cPLA_2$), but not by an inhibitor of calcium-independent PLA_2 ($iPLA_2$). Apocynin also suppressed the increased production of superoxide anions ($O_2^{\cdot-}$) and phosphorylation of $cPLA_2$ induced by $Aβ_{42}$. In addition, hydrolyzed products of $cPLA_2$, arachidonic acid (AA), but not lysophosphatidylcholine (LPC) caused astrocyte membranes to become more molecularly-ordered. These results suggest (1) a direct interaction of $Aβ_{42}$ with cell membranes making them more molecularly-disordered, and (2) $Aβ_{42}$ indirectly makes membranes become more molecularly-ordered by triggering the signaling pathway involving NADPH oxidase and $cPLA_2$.

CHAPTER 1

INTRODUCTION

Increased production of amyloid-β peptides (Aβ) and their deposition as amyloid plaques in brains have been implicated in the pathogenesis of Alzheimer's disease (AD). In fact, the soluble oligomeric form of Aβ is cytotoxic to neurons and glial cells (Selkoe 2002). The cleavage of amyloid precursor protein by α-secretase at the trans-membrane domain demonstrated a hydrophobic property of the peptide at the carboxyl terminal and their ability to bind lipids (Ashley, Harroun et al. 2006). Studies also demonstrated the ability of Aβ to perturb membranes and alter synaptic functions, such as calcium signaling, enzyme activity, and lipid transport (Arispe, Rojas et al. 1993; Hartmann, Eckert et al. 1994; McLaurin and Chakrabartty 1996; Sultana and Butterfield 2008). It has also been reported that the alteration of synaptosomal membrane fluidity induced by Aβ may underline impairment in memory and learning (Hashimoto, Hossain et al. 2006). However, the mechanism underlying the effects of Aβ on cell membrane properties has yet to be elucidated. In this study, we demonstrate that phospholipase A_2 (PLA_2) is involved in the mechanism underlying the effects of $A\beta_{42}$ oligomers on cell membrane phase properties.

Phospholipases A_2 (PLA_2) are enzymes catalyzing the cleavage of fatty acids from the *sn*-2 position of phospholipids to produce free fatty acids and lysophospholipids (Dennis 1994; Balsinde, Balboa et al. 1999). PLA_2 are generally grouped into three major types, the Ca^{2+}-dependent group IV cytosolic PLA_2 ($cPLA_2$), the Ca^{2+}-independent group VI PLA_2 ($iPLA_2$) and the Ca^{2+}-

dependent group II secretory PLA_2 ($sPLA_2$) (Murakami and Kudo 2002). PLA_2 not only plays a role in maintenance of cell membrane integrity, they are also critical in regulating the release of arachidonic acid (AA), a precursor for eicosanoids (Murakami and Kudo 2002). PLA_2 has been implicated in a number of neurodegenerative diseases including AD (Farooqui and Horrocks 2006; Forlenza, Schaeffer et al. 2007). A marked elevation in $cPLA_2$ in the nucleus basalis and hippocampal regions of the AD brain has been observed (Stephenson, Rash et al. 1999; Farooqui, Ong et al. 2003; Farooqui, Ong et al. 2003). Excessive PLA_2 activity disrupts membrane fluidity and alters composition and subsequently, activity of membrane-dependent proteins, such as Na-K-ATpase, beta2- and alpha2-adrenergic receptors, norepinephrine and serotonin uptake, and imipramine binding (Hibbeln, Palmer et al. 1989). Our previous study demonstrated that $A\beta_{42}$ oligomers increase localization of phosphorylated $cPLA_2$ with mitochondria in astrocytes, and that activations of both $cPLA_2$ and $iPLA_2$ are the key steps in $A\beta_{42}$ oligomer-induced mitochondrial dysfunction in astrocytes (Zhu, Lai et al. 2006). Therefore, it is reasonable to hypothesize that oligomeric Aβ perturbs cell membrane properties not only through direct interaction with or insertion into cell membranes, but also through stimulating signaling pathways involving PLA_2.

In this study, we applied fluorescence microscopy of an environmentally sensitive probe, Laurdan, integrated into membranes of astrocytic DITNC cells, to characterize the changes in membrane molecular order induced by oligomeric

$A\beta_{42}$. Inhibition studies demonstrated the involvement of NADPH oxidase and $cPLA_2$ in $A\beta_{42}$-induced membrane molecular order changes in DITNC cells.

CHAPTER 2

BACKGROUND

2.1 Alzhiemer's Disease

Alzhiemer's Disease(AD) was first described by Dr. Alois Alzheimer in 1906. AD is a progressive neurodegenerative disease typified by memory loss and general cognitive decline, and is currently incurable. AD is the 7th leading cause of death in the U.S., and as of 2010 more than 5 million Americans suffer from AD, making it the most common form of dementia. The resultant measureable financial impact is over 170 billion dollars annually, with 10.9 million unpaid caregivers contributing to a much larger actual socio-economic burden(Alz. Association Facts and Figures 2010). Clearly, insight into the causative mechanisms of AD, and subsequent development of new therapies are desperately needed.

The pathological lesions in AD are amyloid plaques and neurofibrillary tangles. While, neurological and cognitive testing can help predict an AD diagnosis, a true determination can only be had after post-mortem evaluation of brain tissue for the presence of these lesions. Amyloid plaques result from the aggregation of amyloid-beta-peptide (Aβ), while neurofibrillary tangles are caused by the hyper-phosphorylation of microtubule-associating Tau protein, forming insoluble aggregates . Much of current AD research has focused on the action of small soluble oligomers of amyloid-beta-peptide 1-42($A\beta_{42}$), and $A\beta_{42}$ has been strongly implicated as a primary neurotoxic species in AD . As such,

the present study focuses on the role of $A\beta_{42}$ in alteration of membrane properties, as well as activation of downstream pathways relevant to AD.

2.2 Amyloid β-peptide and phospholipase A_2 in AD

Amyloid plaques, the pathological hallmark of AD, are composed of aggregated Amyloid β-peptide (Aβ), a peptide 39-43 amino acids long, which is formed by the cleavage of the integral trans-membrane amyloid precursor protein(APP). Processing of APP is facilited by the α, β, and γ secretases, which specifically cleave APP at their respective active sites to form product peptides of varying length and sequence, depending on which secretases act and in what order. Sequential cleavage by the β and γ-secretases results in the formation of Aβ, which is referred to as amyloidogenic APP processing(Vassar 2004). While large, insoluble aggregates of Aβ are the hallmark lesions of the disease, soluble oligomers of Aβ have been strongly implicated as a primary neurotoxic species in AD through induction of oxidative stress (OS), initiation of pro-inflammatory pathways, disruption of cell membranes, and alteration of calcium homeostasis(Mattson, Cheng et al. 1992; Arispe, Rojas et al. 1993; Demuro, Mina et al. 2005). In fact, amyloid-β peptide 1-42 ($A\beta_{42}$) induces generation of reactive oxygen species (ROS) through activation of NADPH oxidase in both astrocytes and neurons(Zhu, Lai et al. 2006; Shelat, Chalimoniuk et al. 2008). Excess ROS in cells disrupts normal cell functions, including intracellular signaling and cytoskeleton organization, as well as directly damaging lipids and DNA.

In this regard, our group has previously reported that $A\beta_{42}$ induces oxidative stress through NADPH oxidase, and mitochondrial dysfunction in astrocytes mediated by the activation of phospholipase A_2(PLA_2)(Zhu, Lai et al. 2006). PLA_2s are a family of phospholipases which catalyze the cleavage of phospholipids at the *sn*-2 position releasing free fatty acids and lysophospholipids. PLA_2s are important regulators of normal lipid metabolism, as well as the inflammatory response through their specific generation of the eicosanoid precursor arachidonic acid(AA). The activity of pLA_2s is highly regulated though both phosphorylation by the mitogen activated protein kinase (MAPK) pathways, as well as for some species through intra-cellular calcium concentration.

Interestingly, it has been reported that cytosolic-phospholipase A_2($cPLA_2$) is upregulated in AD brain(Lukiw and Bazan 2000). $cPLA_2$ is Ca^{2+} dependent, and after phosphorylation at ser^{505} by MAPK, $cPLA_2$ will (in the presence of sufficient Ca^{2+}) translocate to the plasma membrane, where it can interact with phospholipids releasing AA, and modifying the local properties of the membrane(Leslie 1997). It has been reported that NADPH oxidase activation is dependent on the local membrane environment, and our group has previously shown the membrane subunit of NADPH oxidase, gp91*phox*, is predominately localized in highly molecularly-ordered domains in astrocytes(Zhu, Hu et al. 2009). These findings lead to the hypothesis proposed in this work that $A\beta_{42}$ oligomers directly alter membrane properties, induce oxidative stress in

astrocytes through activation of NADPH oxidase, and activate $cPLA_2$ resulting in downstream modification of membrane physical properties.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified Eagle medium (DMEM), F12 medium, Iscoves's modified Dulbecco's medium (IMDM), phosphate-buffered saline (PBS), Tris-buffered saline (TBS), trypsin-EDTA, streptomycin-penicillin, and fungizone were from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) was from US Bio-Technologies (Parkerford, PA). Methyl arachidonyl fluorophosphonate (MAFP) and (s)-bromoenol lactone (BEL) were from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal $cPLA_2$ and phosphorylated-$cPLA_2$ (Ser505) antibodies were from Cell Signaling Technology (Beverly, MA). 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), and dihydroethidium (DHE) were from Invitrogen (Eugene, OR). Bovine serum albumin (BSA), poly-D-lysine, arachidonic acid (AA), lysophosphatidylcholine (LPC), phorbol myristate acetate (PMA) and apocynin (Apo) were from Sigma (St. Louis, MO). $A\beta_{1\text{-}42}$ ($A\beta_{42}$) and $A\beta_{42\text{-}1}$ peptides were purchased from American Peptide Company, Inc (Sunnyvale, Calif.).

3.2 Cell Culture

Immortalized rat astrocytes (DITNC) were purchased from ATCC (Rockville, Md., USA). Cells were maintained at 37°C in the CO_2 humidified

incubator. DMEM culture medium supplemented with 10% of fetal bovine serum, 1% of penicillin/streptomycin (100 units/100 mg/ml) and 1% of fungizone (250 mg/ml) was fed to the cells every 48 hr. When cells grew to confluency, they were subcultured to new flasks or dishes accordingly. In preparation for subculture, cells were washed twice with PBS and then incubated with 3 ml of 0.05% Trypsin-EDTA at 37°C for 5 min. Culture medium was added to stop the trypsin-EDTA enzyme reaction and cells were then sedimented by centrifugation at 2,000 rpm for 10 min. After discarding the supernatant, cells were resuspended in the same culture medium mentioned above and seeded to desired dishes or plates according to experiment condition at $3 \times 10^4/cm^2$ density. Experiments were performed when cells achieved approximately 70-80% confluency.

3.3 Preparation of $A\beta_{42}$ oligomers

$A\beta_{42}$ was obtained from American Peptides and its oligomeric form was prepared according to the protocol described by (Dahlgren, Manelli et al. 2002). Briefly, the peptide (1 mg) in powder form was dissolved in 200 µL of hexafluoro-2-propanol (HFIP) and the solution was aliquoted into Eppendorf tubes. Organic solvent was removed using a speed vacuum apparatus. The Aβ film left in the tube was re-suspended in DMSO and further diluted in Ham's F12 medium to make a 100 µM solution. The solution was incubated at 4^oC for 24 hrs prior to use. As negative control, $A\beta_{42\text{-}1}$ (American Peptides) was processed similarly.

Electrophoretic analysis of $A\beta_{42}$ indicated a similar profile with oligomers in the preparation as described by (Dahlgren, Manelli et al. 2002).

3.4 Cell Assays

DITNC cells were grown on lysinated glass coverslips to approximately 60% confluency. Before the addition of necessary reagents, cells were incubated with a serum-free DMEM overnight. Cells with/without pretreatment of inhibitors (e.g. MAFP, BEL and apocynin in DMSO) for 1 h were treated with the desired concentration of stimulators (e.g. oligomeric $A\beta_{42}$, and PMA in PBS; AA and LPC in EtOH) at 37°C.

3.5 Preparation of laurdan-labeled lipid vesicles

Lipids in rat brains were extracted following the procedure described by Zhang and Sun (Zhang and Sun 1995). Briefly, brain tissue was homogenized in 10 ml of PBS and 40 ml of chloroform-methanol (2:1 v/v) was added. The mixture was centrifuged at 2000 g for 10 minutes. The lower organic phase containing the lipids was filtered through a Pasteur pipette column packed with glass wool and anhydrous Na_2SO_4. The lipid extract was collected and stored at -20ºC until use.

To prepare Laurdan-labeled vesicles, ~0.001 mol% of Laurdan was added to brain lipids dissolved in chloroform-methanol (2:1 v/v) solvent. The

preparation of vesicles was accomplished by electroformation as described in (Lee, Bermudez et al. 2001).

3.6 Fluorescence microscopy of laurdan-labeled cells and visualization of membrane molecular order

A membrane environmentally sensitive probe, Laurdan, was applied to characterize the molecular order of plasma membranes in cells. This compound has been applied to detect phase transitions of different lipid systems as well as natural membranes (Parasassi, Di Stefano et al. 1992; Parasassi, Gratton et al. 1997). Based on the parameters introduced by Parasassi et al. (Parasassi, Di Stefano et al. 1992; Parasassi, Gratton et al. 1997), generalized polarization (GP) of Laurdan is defined as: $GP = (I_B-I_R)/(I_B+I_R)$, where I_B and I_R are the intensities at 440 nm and 490 nm respectively with a fixed excitation wavelength of 350 nm. A higher GP indicates a more molecularly-ordered membrane, and a lower GP indicates a more molecularly-disordered membrane.

After treatment at 37°C, cells attached on glass cover slips were washed twice with PBS and incubated with DMEM containing 1% Laurdan for 15 min. Excess Laurdan was removed by washing cells three times with PBS. The cover slips were then transferred to a Bioptechs FCS2 Focht live-cell thermo chamber (Butler, PA) filled with phenol red free DMEM medium, in which temperature was maintained at 37^oC. Laurdan has been proved to preferentially integrate into plasma membranes with this protocol (Zhu, Tan et al. 2005).

Fluorescence microscopy of Laurdan labeled cells was accomplished with a Nikon TE2000-U inverted microscope with a 60X, NA 1.4 oil immersion objective lens. A dual view micro-imager was installed to the emission port of the microscope, which allowed the acquisition of a pair of fluorescent images from a sample simultaneously at the emission wavelengths of 446 and 499 nm with a 46 nm bandwidth. These pairs of fluorescent images were used to calculate the local GP values of the cells in the pixel basis. The local GP values were then used to reconstruct a GP mapped image for direct visualization of local membrane phase properties in cells.

To compute spatially-distributed GP(x,y) for constructing GP-mapped images by pseudo color representation of GP(x,y), paired images obtained at emission wavelengths of 446 and 499 nm were processed as follows: (1) convolution of both images with a Gaussian kernel to reduce noise; (2) computation of the denominator by pixel-wise addition of both images; (3) obtaining thresholds of the result using Otsu's method to separate Laurdan-stained regions from the background; and (4) computation of GP(x,y) on a pixel-by-pixel basis with the additional condition that the resulting pixel is zero if the thresholded denominator is zero.

3.7 Measurement of superoxide anion production in DITNC cells

Dihydroethidium (DHE) was used to determine superoxide anion ($O_2^{\cdot -}$) production following a modified protocol as described by Chapman et al. (Chapman, Sinclair et al. 2005). Upon contact with superoxide anions,

oxoethidium, a highly fluorescent product from the oxidative reaction of DHE, binds to DNA, causing an increase in fluorescent intensity of the cell nuclei. In this study, DITNC cells were grown on 8-well chambers and serum starved overnight. Cells were rinsed twice with warm serum-free and phenol red-free (PR-Free) DMEM and incubated with DHE (5μM) in PR-free DMEM at 37°C for 30 min. After removing excess DHE, cells were suspended in PR-free DMEM containing $A\beta_{42}$, PMA, apocynin (Apo), Apo + $A\beta_{42}$, and Apo + PMA for 1 h prior to taken images.

Fluorescence microscopy for DHE was performed with a Nikon TE-2000 U fluorescence microscope and a 40X, NA 0.95 objective lens. Images were acquired using a cooled CCD camera controlled by a computer running a MetaVue imaging software (Universal Imaging, PA). The fluorescence excitation source was controlled with a Uni-Blitz mechanical shutter. For image acquisition, a short exposure time (100 msec) and low intensity excitation light were applied to minimize photo-bleaching. The fluorescent intensity of DHE in each cell was quantified by integrating the pixel-intensity of the cell. Background subtraction was done for each image prior to the quantification of DHE intensity of cells. Each experiment was repeated three times for statistical data analysis.

3.8 Western blot analysis

DITNC cells were cultured in 60-mm dishes until 90% confluent. After treatment as described above, cells were washed with ice-cold PBS twice and

200 µl cell lysate medium (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue) were added. After collecting the cell lysate, protein concentrations were determined by the Bradford assay (Bradford 1976). Equivalent amounts of protein for each sample were applied to 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (0.45 micron, Bio-Rad). Membranes were incubated in Tris-buffered saline, pH 7.4 with 0.5% Tween 20 (TBS-T) containing 5% non-fat milk for 1 h at room temperature. The blots were reacted with primary rabbit anti-$cPLA_2$ or anti-p-$cPLA_2$ or anti-lamin A/C (1:1000; Cell Signaling) at 4ºC overnight with gentle shaking. After washing with TBS-T, the membranes were incubated with goat anti-rabbit IgG-HRP (1:2000; Sigma) for 1 hr at room temperature and then washed 3× with TBS-T. Proteins were detected and visualized by enhanced chemiluminescence using a SuperSignal West Pico Chemiluminescent Detection Kit (Pierce Biotechnology, Inc). $cPLA_2$ and p-$cPLA_2$ bands were detected at 105 kDa.

3.9 Statistical analysis

Data are presented as mean ± SD from at least three independent experiments. Comparisons between groups were made with one-way ANOVA, followed by Bonferroni's post hoc tests. Comparison between two groups was made with paired *t* test. Values of $p < 0.02$ are considered statistically significant.

CHAPTER 4

RESULTS

4.1 Oligomeric $A\beta_{42}$ induced temporal membrane biphasic changes in DITNC cells through NADPH oxidase

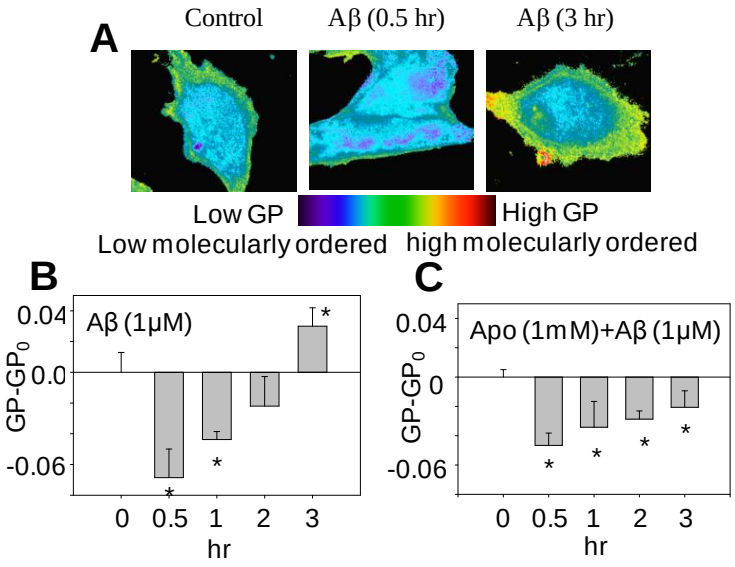
We applied the fluorescence microscopy of Laurdan integrated into plasma membranes of astrocytic DITNC cells to study the possible changes in membrane phase properties induced by oligomeric $A\beta_{42}$. Since Laurdan possesses both an electron donor and an electron acceptor, fluorescent excitation can induce a large excited-state dipole. This strong dipole tends to locally align the surrounding molecules (e.g. water), which dissipates a small fraction of the excited state energy and produces a red shift in the emission spectrum. A molecularly-disordered membrane allows more water molecules to partition into the membrane core, which is manifested by a red shift of Laurdan's emission maximum (Parasassi, De Stasio et al. 1990; Lee, Law et al. 2001). To quantify this shift, Gratton and co-workers (Parasassi, De Stasio et al. 1990) have defined the generalized polarization (GP) which was applied to observe phase transitions of different lipid membranes (Parasassi, Ravagnan et al. 1993; Parasassi, Giusti et al. 1994) as well as cell membranes (Waschuk, Elton et al. 2001; Zhu, Tan et al. 2005). A higher GP value indicates a more molecularly-ordered membrane, while a lower GP value indicates a more molecularly-disordered membrane. It has also been proved that once it is integrated into the plasma membranes of astrocytes, it would be unlikely to diffuse further into the

intracellular organelles due to the hydrophobic properties of Laurdan (Zhu, Tan et al. 2005).

Pseudo-colored GP-mapped images were reconstructed for direct observation of changes in GP values at different time points in cells after treatment with 1 μM of oligomeric $A\beta_{42}$ (Fig. 1A). In our data analysis, we plotted $GP\text{-}GP_o$, where GP_o is the GP of control experiment (i.e. no $A\beta_{42}$ treatment). Therefore, $GP\text{-}GP_o$ of the control is always zero, serving as a common reference datum. Fig. 1B shows that $A\beta_{42}$ oligomers made cell membranes become more molecularly-disordered within 30 mins, as indicated by negative $GP\text{-}GP_o$ values. However, these $GP\text{-}GP_o$ values became more positive with time and was more molecularly-ordered compared to the control at 3 hr after the oligomeric $A\beta_{42}$ treatment, as indicated by a positive $GP\text{-}GP_o$ value. In order to test whether oxidative stress induced by NADPH oxidase plays a role in the time-dependent changes in GP values, apocynin, was used to pretreat cells for 1 hrs followed by the treatment with oligomeric $A\beta_{42}$. In the presence of apocynin, the $GP\text{-}GP_o$ values were negative, indicating more molecularly-disordered membranes (Fig. 1C). These results suggest that the activation of NADPH oxidase is required for oligomeric $A\beta_{42}$ to make DITNC cell membranes become more molecularly-ordered.

The treatment of $A\beta_{42}$ for cells not only affected the overall (macroscopic) changes in GP (i.e. $GP\text{-}GP_o$), but it also altered the distribution of GP. Fig. 1D (*upper*) shows GP ranging from -0.4 to 0.4 with a peak at GP ≈ -0.14. After treatment with $A\beta_{42}$ for 30 min, the population of high GP values was decreased,

and the peak was found at GP ≈ -0.25 (Fig. 1D, *middle*). However, after treatment with $A\beta_{42}$ for 3 hr, the population of high GP increased with two major peaks at GP = -0.06 and GP = 0.1, which was suppressed by Apo (data not shown).



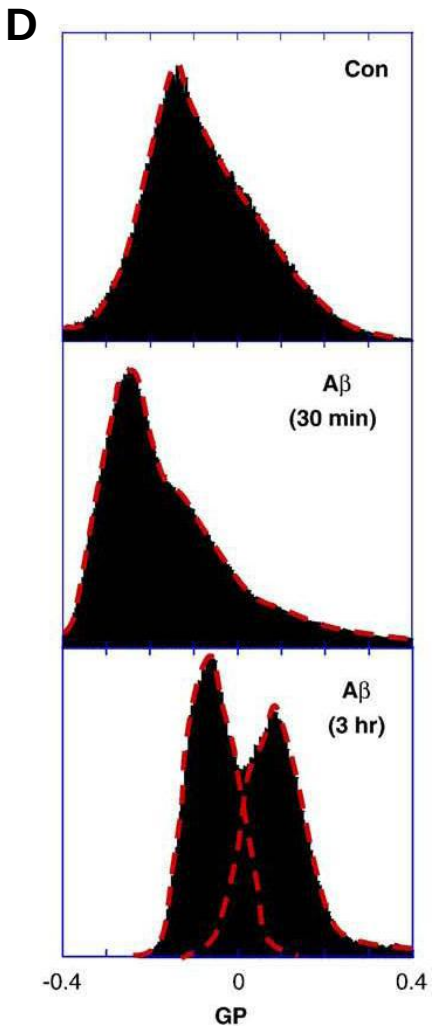


Fig. 1. $A\beta_{42}$ induced temporal biphasic changes of membrane molecular order in DITNC cells.

(A) Pseudo-colored GP-mapped images of DITNC cells were reconstructed based on the GP value of each pixel. Control (left); a GP-mapped image taken at 0.5 h after treatment with 1 μM of $A\beta_{42}$ (middle); a GP-mapped image acquired at 3 h after treatment with 1 μM of $A\beta_{42}$ (right).

(B) The GP–GPo (GPo=−0.0030±0.016, is the GP of control sample without $A\beta_{42}$ treatment at time=0) exhibited a negative value at 0.5 h after $A\beta_{42}$ treatment to cells, indicating more molecularly-disordered membranes, but a positive value at 3 h, indicating more molecularly-ordered membranes.

(C) The GP–GPo (GPo=0.074±0.0051, is the GP of control sample treated with Apocynin (1 mM) in DMSO) exhibited only negative values in cells pretreated with apocynin (1 mM) for 1 h followed by $A\beta_{42}$ (1 μM) treatment. (D) Changes of GP distribution in response to the treatment of cells with $A\beta_{42}$; without treatment (upper); treatment with $A\beta_{42}$ for 30 min (middle); treatment with $A\beta_{42}$ for 3 h (lower).

(D) Changes of GP distribution in response to the treatment of cells with Aβ; without treatment (upper); treatment with $A\beta_{42}$ for 30 min (middle); treatment with $A\beta_{42}$ for 3 h (lower).

4.2. Oligomeric $A\beta_{42}$ caused artificial membranes made of rat brain lipid extract to become more molecularly-disordered

To rule out the factors of cellular processes contributing to $A\beta_{42}$-mediated changes in the molecular order of membranes, we examined changes in artificial membranes made of rat brain lipid extract. Fig. 2A showed that oligomeric $A\beta_{42}$ caused artificial membranes to become more molecularly-disordered, as indicated by negative $GP\text{-}GP_o$ values. For a negative control, we examined if the reversed amino acid sequence of Aβ (i.e. $A\beta_{42\text{-}1}$) affects the artificial bilayer membranes made of brain lipid extract. Fig. 2B shows that $A\beta_{42\text{-}1}$ only induced small changes in the molecular order of artificial membranes, and the changes were experimentally insignificant. These results are consistent with the notion that $A\beta_{42}$ directly interacts with membranes making them more molecularly disordered (Eckert, Cairns et al. 2000). These results also lead to the hypothesis that $A\beta_{42}$ makes cell membranes become more molecularly-ordered through intracellular signaling pathways, which probably requires a long time (as shown in Fig. 1, it took more than 3 h for cell membranes to become more molecularly-ordered)

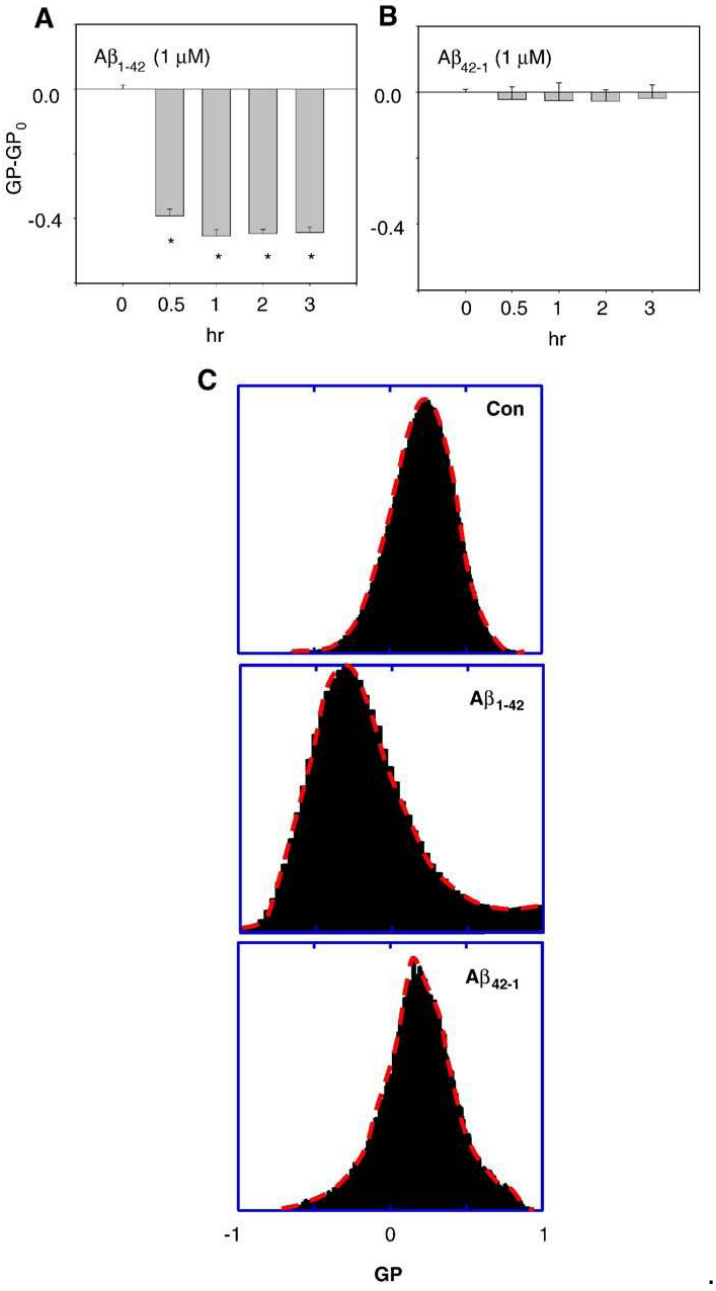


Fig. 2. Oligomeric $A\beta_{42}$ caused artificial membranes made of rat brain lipid extract to become more molecularly-disordered

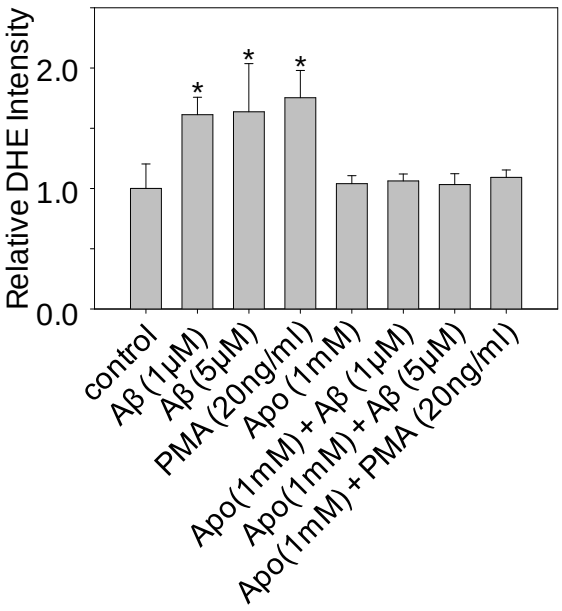
(A) The GP–GPo (GPo=−0.24±0.085, is the GP of control sample without treatment at time=0) of Laurdan integrated in vesiclemembranes of rat brain lipid extract after $A\beta_{42}$ treatment exhibited only negative values, indicating more molecularlydisorderedmembranes.

(B) The reversed amino acid sequence of Aβ, $A\beta_{42-1}$, had no effect on the GP–GPo value. (*p< 0.02) (C) The GP distribution after treatments with $A\beta_{1-42}$ and $A\beta_{42-1}$; without treatment (upper); treatmentwith $A\beta_{1-42}$ for 1 h (middle); treatment with $A\beta_{42-1}$ for 1 h (lower).

4.3. Oligomeric $A\beta_{42}$ increased superoxide anion production and phosphorylation of cPLA2 through NADPH oxidase

To test the hypothesis that NADPH oxidase and $cPLA_2$ are involved in the $A\beta_{42}$-induced membrane biphasic changes in DITNC cells, we first examined whether oligomeric $A\beta_{42}$ increases superoxide anion ($O_2^{\cdot-}$) production and phosphorylation of $cPLA_2$ through NADPH oxidase in DITNC cells. We found that oligomeric $A\beta_{42}$ increased the production of $O_2^{\cdot-}$ in DITNC cells, as indicated by increased fluorescent intensity of DHE. The increase of DHE intensity was suppressed by the inhibitor of NADPH oxidase, apocynin (Fig. 3). For a positive control, we treated cells with the phorbol ester (PMA), which has been reported to increase the production of $O_2^{\cdot-}$ in astrocytes through NADPH oxidase (Abramov, Jacobson et al. 2005).

Western blot analysis of phosphorylated $cPLA_2$ (p-$cPLA_2$) shows that oligomeric $A\beta_{42}$ (1 μM) increased the immunoreactivity of p-$cPLA_2$ in DITNC cells, and this effect was suppressed by apocynin (Fig. 4). Since apocynin was dissolved in DMSO, DMSO alone was not able to trigger phosphorylation of $cPLA_2$. These results are consistent with our previous reported with primary astrocytes (Zhu, Lai et al. 2006). However, the response of DITNC cells to $A\beta_{42}$ treatment is slower as compared with that of primary astrocytes.



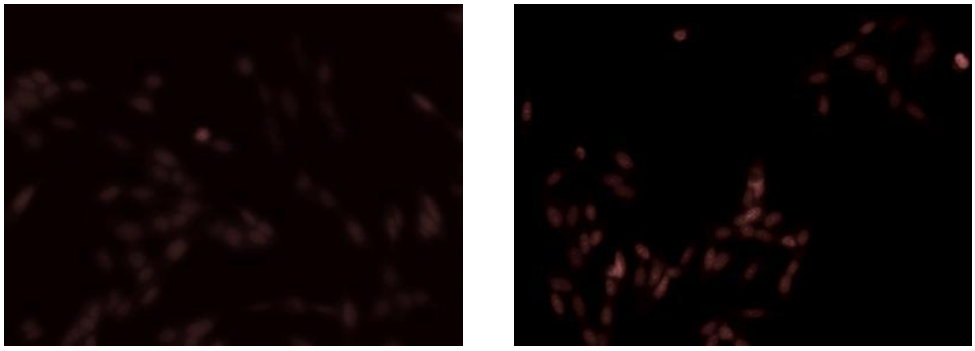


Fig. 3. Oligomeric Aβ42 caused artificial membranes made of rat brain lipid extract to become more molecularly-disordered

The fluorescent intensity of DHE was measured as an indicator of the superoxide anion ($O_2^{\cdot -}$) production level in cells. (*$p<0.02$) Apocynin suppressed the $O_2^{\cdot -}$ production induced by $A\beta_{42}$, supporting that $A\beta_{42}$ increases $O_2^{\cdot -}$ production in DITNC cells through NADPH oxidase.

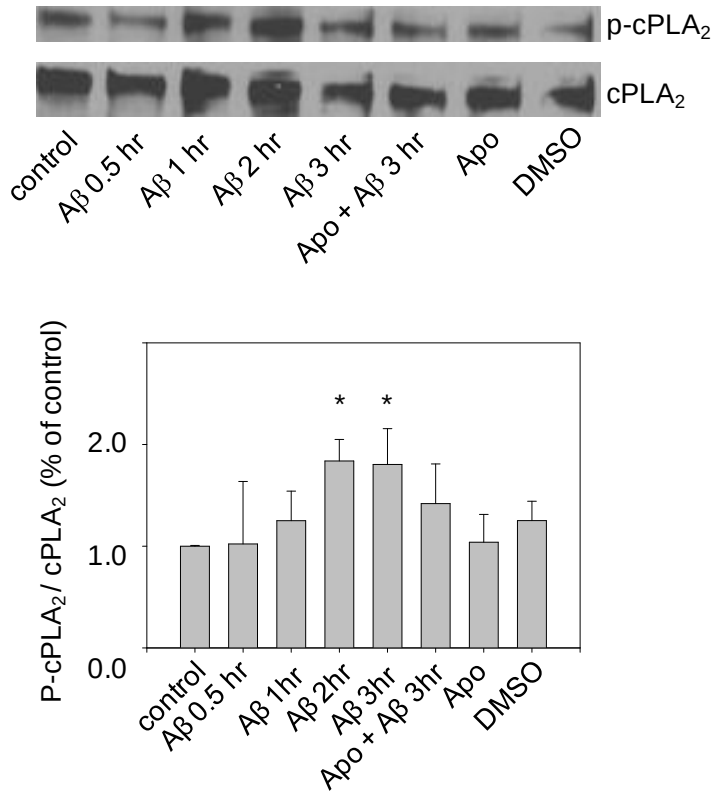


Fig. 4. $A\beta_{42}$ induces phosphorylation of $cPLA_2$ in DITNC cells

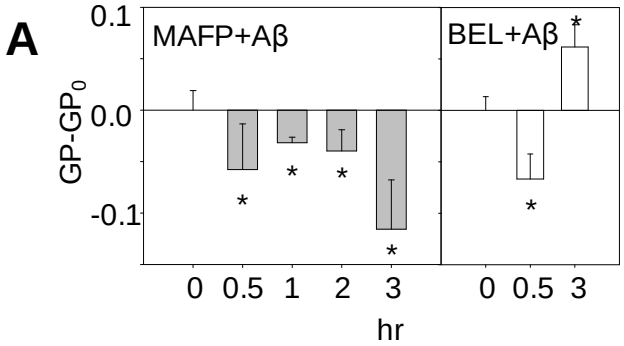
DITNC cells were stimulated with Aβ42 (1 μM) for different incubation times and were subjected to Western blot analysis with anti-p-cPLA2 and anti-cPLA2 antibody. Results were from one representative experiment (upper). Repeated experiments gave similar results. For each sample, the relative intensity of phosphorylated cPLA2 was normalized by the total cPLA2 (lower). Values are the mean of three independent experiments. (*p<0.02)

4.4. cPLA2 was the key enzyme involved in $A\beta_{42}$-induced membrane biphasic changes

Inhibitors of PLA_2 were applied to study the involvement of PLA_2 in $A\beta_{42}$-induced membrane changes in DITNC cells. When the inhibitor of $cPLA_2$ and calcium-independent PLA_2 ($iPLA_2$), methyl arachidonyl fluorophosphonate (MAFP), was added to cells and followed by treatment with $A\beta_{42}$, the GP-GP_o

values only exhibited negative numbers (Fig. 5, *left*), indicating more molecularly-disordered membranes, and the ability of $A\beta_{42}$ to make membrane become more molecularly-ordered as indicated by a positive $GP\text{-}GP_o$ value at 3 hr in Fig. 1B was totally abrogated in Fig. 5, *left*. However, the specific inhibitor of $iPLA_2$, bromoenol lactone (BEL), was incapable of suppressing the membrane biphasic changes induced by oligomeric $A\beta_{42}$ (Fig. 5, *right*), which ruled out the role of $iPLA_2$. These results suggested that $cPLA_2$ (but not $iPLA_2$) was the key enzyme involved in making membranes become more molecularly-ordered as observed in the biphasic changes of DITNC cell membranes induced by oligomeric $A\beta_{42}$.

The distribution of GP in Fig. 5B, (*lower right*) shows that BEL was incapable of suppressing the increased population of high GP domains with a peak at GP ≈ 0.1, whereas MAFP totally suppressed the formation of high GP domains (Fig. 5B, *lower left*). These results directly demonstrate that $A\beta_{42}$ makes membranes become more disordered, but also activates $cPLA_2$, resulting in the formation of high GP domains.



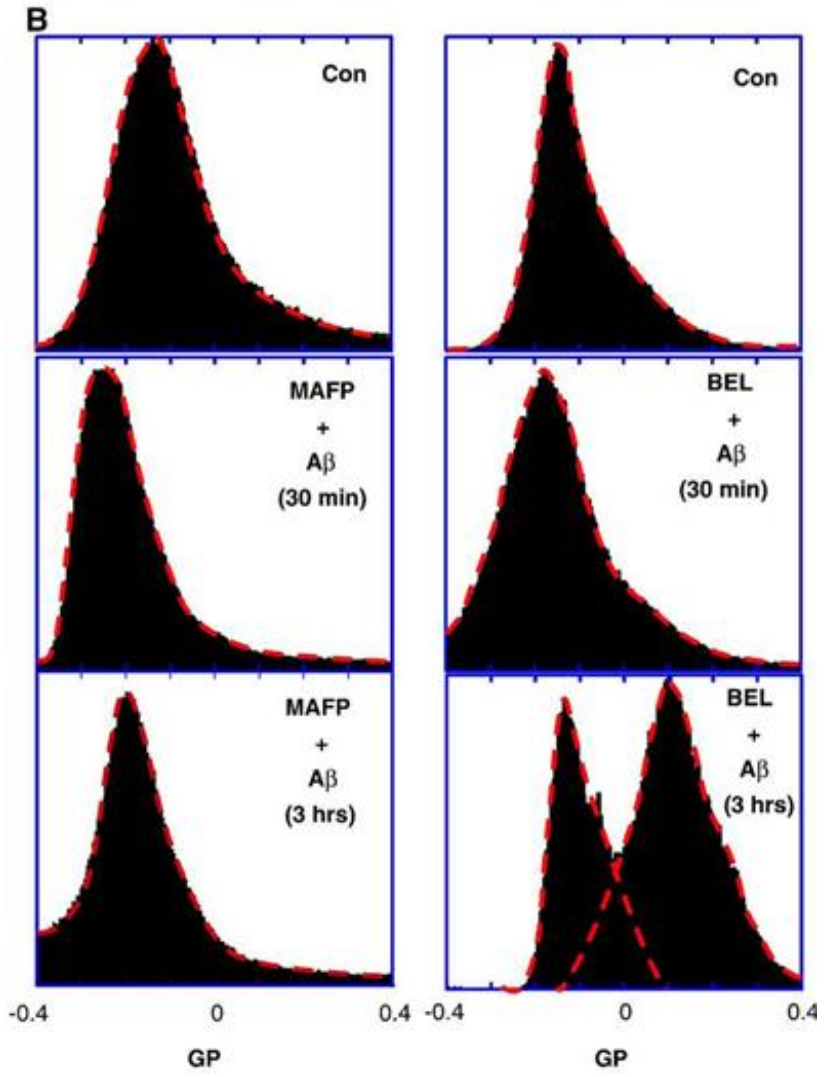


Fig. 5. Effects of PLA_2 inhibitors on $A\beta_{42}$ induced changes in membrane molecular order

(A)The membrane order changes induced by Aβ42 (1 μM) was totally suppressed by MAFP (5 μM) as indicated by GP–GPo values (GPo=0.074±0.06, is the GP of control cells pretreated with 5 μM of MAFP in DMSO) exhibiting negative numbers only (left), but was not suppressed by BEL (5 μM) (GPo=−0.059±0.018, is the GP of control cells pretreated with 5 μM of BEL in DMSO) (right).

(B) GP distribution after treatment with Aβ42 for cells pretreated with MAFP (left) and BEL (right).

4.5. The hydrolyzed products of cPLA2 made cell membranes become more molecularly-ordered

When $cPLA_2$ is activated, it targets and hydrolyzes phospholipids in cell membranes, resulting in hydrolyzed products, such as arachidonic acid (AA) and lysophosphatidylcholine (LPC). Since these hydrolyzed products of $cPLA_2$ may also contribute to the change of membrane molecular order induced by oligomeric $A\beta_{42}$ in DITNC cells, we applied the fluorescence microscopy of laurdan integrated in membranes of DITNC cells to study possible membrane changes caused by AA and LPC. We found that AA made membranes of DITNC cells more molecularly-ordered, as indicated by positive $GP\text{-}GP_o$ values; whereas LPC did not cause significant changes of membranes. Similarly, LPC did not cause any major effect on the GP distribution (Fig. 6, *right*). However, AA made significant changes in the distribution of GP (Fig. 6, *left*) and produced two major populations with their peaks at GP ≈ -0.1 and GP ≈ 0.025.

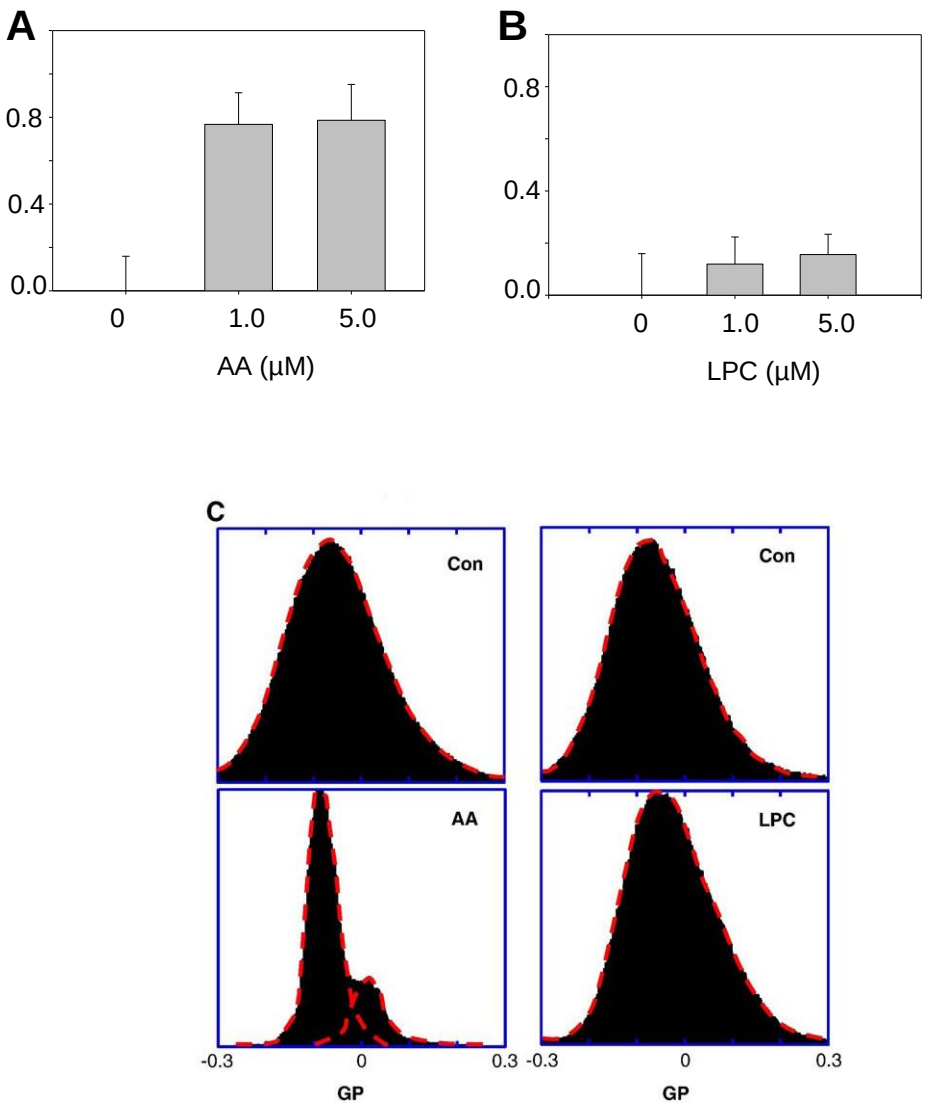


Fig. 6. Effects of hydrolyzed products of cPLA2 on membranes in DITNC cells.

(A) The treatment with arachidonic acid (AA) for 1 h caused cell membranes to become more molecularly-ordered, as indicated by positive GP–GPo values.

(B) The treatment with lysophosphatidylcholine (LPC) for 1 h did not cause a significant change in GP. (*p<0.02) (GPo=−0.038±0.016 is the GP of control cells treated with ethanol, as both AA and LPC were dissolved in ethanol.)

(C) GP distribution of DITNC cell membranes treated with AA (left) and LPC (right).

CHAPTER 5

DISCUSSION

Applying fluorescence microscopy of Laurdan integrated into membranes of astrocytic DITNC cells and artificial membranes made of rat brain lipid extract, we demonstrated that both NADPH oxidase and $cPLA_2$ were involved in the $A\beta_{42}$ oligomer-induced temporal biphasic membrane change in DITNC cells. Our data also suggest that a hydrolyzed product of $cPLA_2$, arachidonic acid (AA), contributes to the effect of $A\beta_{42}$ in causing cell membranes to become more molecularly-ordered through the activation of NADPH oxidase and $cPLA_2$.

The absolute changes of GP in artificial membranes made of whole brain lipid extract induced by $A\beta_{42}$ was about 0.4 (Fig. 2A), which was greater than that in $A\beta_{42}$-stimulated DITNC cells, where the maximum absolute change of GP can be estimated from the GP's at time points 0.5 hr and 3 hr to be about 0.1 (Fig. 1B). Fig. 1C also shows that the absolute change of GP in $A\beta_{42}$-stimulated cells pretreated with Apo to inhibit NADPH oxidase and $cPLA_2$ was about 0.04. Generally, the changes of GP in cells induced by $A\beta_{42}$ are smaller, as compared to those in artificial membranes, but they are statistically significant. In fact, the GP changes in DITNC cells measured here are comparable to the GP change (~0.13) in macrophages induced by removal of cholesterol with methyl β-cyclodextrin (mβCD) (Gaus, Gratton et al. 2003). We observed that the absolute GP's of controls (i.e. GP_o) varied with different experiments, which is probably due to different inhibitors (e.g. Apo, MAFP, and BEL) being added to different

controls, as these inhibitors contribute slightly to the emission at the wavelengths of 446 and 499 nm tested in PBS(data not shown).

In fact, a correlation between GP values and raft/non-raft domains has been established using liposomes with equal molar ratios of dioleoylphosphatidylcholine (DOPC), cholesterol, and sphingomyelin by Dietrich et al., 2001 (Dietrich, Bagatolli et al. 2001). Our GP distribution of artificial membranes made of whole brain lipid extract (Fig. 2C, *upper*) is comparable to that reported by Dietrich et al., 2001 (Dietrich, Bagatolli et al. 2001). GP>0.55 and <-0.05 were found to represent membranes in gel and fluid phase, respectively. Moreover, (-0.05<GP<0.25) and (0.25<GP<0.55) represent membranes in liquid-disordered/nonraft domains and liquid-ordered/raft domains, respectively (Dietrich, Bagatolli et al. 2001). Separation of liquid-ordered and liquid-disordered phases has also been shown at GP values between 0.2 and 0.3 (Bagatolli and Gratton 1999; Bagatolli and Gratton 2000). The GP values of cell membranes ranging from -0.4 to 0.4 reported here clearly provide evidence of a coexistence of both liquid-disordered/nonraft and liquid-ordered/raft domains. Interestingly, $A\beta_{42}$-induced $cPLA_2$ activation produced a major population of high GP domains with a peak at GP = 0.1 and a GP range of -0.1 to 0.4 (Fig 1D, *lower*, and Fig. 5B, *right lower*). Since the GP of Lubrol-insoluble membranes at 37°C has been reported to be 0.308 ± 0.126 (Gaus, Gratton et al. 2003), these results suggest that $A\beta_{42}$-induced cPLA activation in DITNC cells increases raft heterogeneity and phase separation in membranes. The distributions of GP (Fig 1D, *lower*, and Fig. 5B, *right lower*) observed in $A\beta_{42}$-induced $cPLA_2$ activation

are similar to those reported GP distribution in macrophage membranes at 37°C (Gaus, Gratton et al. 2003). Since macrophages are relatively active immune cells in which $cPLA_2$ can be frequently activated (Dinnes, Santerre et al. 2008), we speculate that the GP distributions in macrophage membranes reported by Gaus et al., (Gaus, Gratton et al. 2003) might be affected in part by $cPLA_2$ activation.

In model membranes areas of high curvature exhibit lower GP values than areas of low curvature(Bagatolli and Gratton 1999). However, for cell membranes similar differences in physical arrangement may not account for comparable changes in GP. It has been shown in living macrophages that variable surface morphologies, cell-to-cell contacts, and possible adhesion locations are enriched in high GP domains, and these high GP domains are likely a result of condensed membrane structure as opposed to differences in curvature(Gaus, Gratton et al. 2003). In agreement with these results, our GP-mapped images of $A\beta_{42}$-stimulated DITNC cells (Fig 1A) show an increase in high GP domains at cell edges, implicating local compositional changes, rather than differences in curvature, as being responsible for the observed increase in high GP domains at these locations.

The release of Aβ from its precursor protein (amyloid precursor protein) and its pathway leading to deposition of amyloid plaques have been regarded to play an important role in the etiology of AD (Selkoe 2002; Selkoe 2006). Recent studies have demonstrated cytotoxic effects of Aβ, especially its aggregation to the oligomeric form (Lambert, Barlow et al. 1998). These soluble diffusible

protofibrils of Aβ have been shown to disrupt membranes, forming calcium channels and subsequently causing increase in calcium influx and triggering cell death pathways (Mattson, Cheng et al. 1992; Arispe, Rojas et al. 1993; Demuro, Mina et al. 2005). These properties of Aβ have induced interest in investigation of how Aβ aggregates impact on cell membrane properties.

Studies with synthetic membranes indicated the ability of oligomeric Aβ to perturb cholesterol containing membranes (Gibson Wood, Eckert et al. 2003; Ashley, Harroun et al. 2006). A number of studies indicated alteration of membrane fluidity by soluble Aβ (Avdulov, Chochina et al. 1997; Muller, Eckert et al. 1998; Mason, Jacob et al. 1999; Eckert, Cairns et al. 2000; Kremer, Pallitto et al. 2000; Chochina, Avdulov et al. 2001; Waschuk, Elton et al. 2001; Yip, Elton et al. 2001). In our study, we investigate cell membrane molecular order changes induced by oligomeric Aβ in DITNC cells, which allow us to address membrane changes not only due to the direct interaction of $A\beta_{42}$ oligomers with membranes, but also due to $A\beta_{42}$ -triggered cellular processes.

Increase in oxidative stress has been regarded to play a role in the development of AD (Nunomura, Perry et al. 2001). It has been reported that $A\beta_{42}$ increases the production of reactive oxygen species (ROS) in astrocytes through the superoxide producing enzyme, NADPH oxidase (Abramov, Canevari et al. 2004; Zhu, Lai et al. 2006). Oxidative stress has also been reported to trigger the MAPK pathways and subsequent activation of PLA_2 (Xu et al., 2002; Zhu et al., 2005). The relationship between $cPLA_2$ and NADPH oxidase under oxidative conditions has also been demonstrated in phagocytic cells (Dana, Leto

et al. 1998; Levy, Lowenthal et al. 2000; Pessach, Leto et al. 2001; Zhao, Bey et al. 2002; Shmelzer, Haddad et al. 2003; Hazan-Halevy, Levy et al. 2005). In neutrophils, $cPLA_2$ was found in cytoplasm and plasma membranes, suggesting multiple role of this enzyme in regulating different subcellular membranes (Levy 2006). In this study, $A\beta_{42}$ oligomers activated NADPH oxidase and $cPLA_2$ in astrocytes, resulting in increased ROS production. In addition, this condition rendered the astrocytes membranes to become more molecularly-ordered.

Immortalized rat astrocytes, DITNC cells, possess the phenotypic characteristics of type 1 astrocytes (Radany, Brenner et al. 1992). Similar to the finding with primary astrocytes (Zhu, Lai et al. 2006), $A\beta_{42}$ induced ROS in DITNC cells and ROS production was inhibited by apocynin, a specific inhibitor for NADPH oxidase (Fig 3). Aβ also increased phosphorylation of $cPLA_2$ in DITNC cells (Fig 4). The ability for apocynin to inhibit Aβ-mediated $cPLA_2$ phosphorylation suggests a link between ROS and $cPLA_2$ activation through NADPH oxidase. However, it is interesting to note that DITNC cells respond to $A\beta_{42}$ stimulation much slower than primary astrocytes. While primary astrocytes took less than 15 min to activate $cPLA_2$ by $A\beta_{42}$ oligomers (Zhu, Lai et al. 2006), DITNC cells took more than 1 hr. The prolonged responsive time of DITNC cells provides an advantage allowing a longer time window for the examination of signaling pathways and measurement of cell membrane property changes. This unique feature provided advantage for using DITNC cell culture as a good cell model for study of astrocytes in neurodegenerative diseases.

Oligomers of $A\beta_{42}$, but not the monomeric peptide, has been reported to insert into cholesterol-containing phosphatidylcholine monolayers with an anomalously low molecular insertion area (Ashley, Harroun et al. 2006). Consistent with the effect of $A\beta_{42}$ oligomers on artificial membranes made of rat brain lipids and membranes of DITNC cells within the first hour reported here, $A\beta_{42}$ oligomers have an enhanced disordering effect on membranes (Eckert, Cairns et al. 2000). However, in membranes from brain lipids, $A\beta_{42}$ monomers render these artificial membranes to become more molecularly ordered (Yip, Darabie et al. 2002). $A\beta_{42}$ assembly at the bilayer surface is dependent on cholesterol, but does not result in bilayer disruption (Yip, Darabie et al. 2002). In our study, we observed a time-dependent transition of the DITNC membrane to become more molecularly-ordered upon incubation with Aβ oligomers. A similar time course for activation of $cPLA_2$ was observed (Fig 4). Our results are consistent with study with erythrocytes in which secretory PLA_2 also renders these membranes to be more molecularly-ordered (Harris, Smith et al. 2001), and are consistent with the finding by others that arachidonic acid makes membranes become more molecularly-ordered (Wilson, Huang et al. 1997).

Increasing evidence has been accumulated to suggest that oligomerization of amyloid peptides leads to alteration of cell membranes and cell signaling pathways. There are other examples in which protein misfolding is an important part of the disease processes. For example, amylin, a molecule in Type II diabetes, also binds and disrupts membranes of insulin-producing β-cells (Janson, Ashley et al. 1999). There is further support that amylin can activate

NADPH oxidase to increase ROS production in β-cells (Janciauskiene and Ahren 2000). In this work we have demonstrated that NADPH oxidase and $cPLA_2$ are involved in the $A\beta_{42}$-oligomer-induced membrane biphasic change in astrocytic DITNC cells. Understanding the mechanism underlying cell membrane alterations induced by $A\beta_{42}$ should prove further our understanding of alteration of primary functions of membrane proteins and in the pathogenesis of AD.

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