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Cytosolic Phospholipase A₂ Facilitates Oligomeric Amyloid- β Peptide Association with Microglia via Regulation of Membrane-Cytoskeleton Connectivity

Tao Teng^a, Li Dong^b, Devin M. Ridgley^a, Shivesh Ghura^c, Matthew K. Tobin^c, Grace Y. Sun^d, Mary Jo LaDu^c, and James C. Lee^{a,1}

^aDepartment of Bioengineering, University of Illinois at Chicago, Chicago, IL 60612

^bDepartment of Bioengineering, University of Missouri, Columbia, MO 65211

^cDepartment of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL 60612

^dDepartment of Biochemistry, University of Missouri, Columbia, MO 65211

Abstract

Cytosolic phospholipase A₂ (cPLA₂) mediates oligomeric amyloid- β peptide (oA β)-induced oxidative and inflammatory responses in glial cells. Increased activity of cPLA₂ has been implicated in the neuropathology of Alzheimer's disease (AD), suggesting that cPLA₂ regulation of oA β -induced microglial activation may play a role in the AD pathology. We demonstrate that LPS, IFN γ , and oA β increased phosphorylated cPLA₂ (p-cPLA₂) in immortalized mouse microglia (BV2). A β association with primary rat microglia and BV2 cells, possibly via membrane-binding and/or intracellular deposition, presumably indicative of microglia-mediated clearance of the peptide, was reduced by inhibition of cPLA₂. However, cPLA₂ inhibition did not affect the depletion of this associated A β when cells were washed and incubated in a fresh medium after oA β treatment. Since the depletion was abrogated by NH₄Cl, a lysosomal inhibitor, these results suggested that cPLA₂ was not involved in the degradation of the associated A β . To further dissect the effects of cPLA₂ on microglia cell membranes, atomic force microscopy (AFM) was used to determine endocytic activity. The force for membrane tether formation (F_{mtf}) is a measure of membrane-cytoskeleton connectivity and represents a mechanical barrier to endocytic vesicle formation. Inhibition of cPLA₂ increased F_{mtf} in both unstimulated BV2 cells and cells stimulated with LPS + IFN γ . Thus, increasing p-cPLA₂ would decrease F_{mtf} , thereby increasing endocytosis. These results suggest a role of cPLA₂ activation in facilitating oA β endocytosis by microglial cells through regulation of the membrane-cytoskeleton connectivity.

Keywords

A β clearance; Alzheimer's disease; cytosolic phospholipase A₂; membrane-cytoskeleton connectivity; microglia

Corresponding Author: James C. Lee; UIC Bioengineering (MC 063), 835 S Wolcott Ave, W100, Chicago, IL 60612; (312) 355-9102.

¹To whom correspondence should be addressed. LeeJam@uic.edu.

Introduction

Growing evidence has shown an important role of microglia in sporadic or late-onset Alzheimer's disease (LOAD) [1]. In addition, increased cytosolic phospholipase A₂(cPLA₂) activity has been observed in AD brains [2,3], although the roles of this enzyme in the pathological expression in AD is not fully understood. The goal of this study is to examine the role of cPLA₂ in soluble oligomeric A β (oA β) association with microglial cells.

As the resident macrophage cells, microglia are key cells responsible for scavenging cell debris, plaques, and damaged neurons and synapses in the brain [4]. In recent years, understanding the mechanism(s) for the microglia-mediated clearance of A β has become an important direction of AD research. A β phagocytosis by microglia has been shown to be governed by a range of receptors [5]. For example, macrophage scavenger receptor 1 (SCARA1) [6,7], CD36 [8-10], and a functional triggering receptor expressed on the myeloid cells 2 protein (TREM2) [11-14] facilitate A β uptake. However, some receptors, such as the CD14-Toll-like receptors (TLRs) [15,16], and CD33, a member of the sialic acid-binding immunoglobulin-like lectins (SIGLECS) family [17,18], reduce the ability to phagocytose A β . Although receptor-mediated A β phagocytosis has been extensively studied, how A β -triggered cellular pathways that determine oA β uptake by microglia are still not fully understood.

Although increased cPLA₂ activity has been implicated in AD [2] and A β has been shown to activate cPLA₂ in microglia [2,19,20], the role of cPLA₂ in alterations of microglial functions related to AD pathophysiology are not fully understood. There is evidence showing oA β causes cellular membranes to become more molecularly-ordered in astrocytes, and this process involves activation of cPLA₂ [19]. cPLA₂ has also been found to mediate actin rearrangements [21,22]. Since endocytosis is both a biochemical and a mechanical process (i.e. mechanochemical processes) governed in parts by the membrane-cytoskeleton connectivity [23], we further tested the hypothesis that cPLA₂ plays a role in endocytosis of oA β in microglia through its effects on membrane-cytoskeleton connectivity.

In this study, we demonstrate that phosphorylation of cPLA₂ in microglial cells is essential in maintaining oA β association with microglial cells, possibly via membrane-binding and/or intracellular deposition. However, cPLA₂ activation is not involved in the degradation of the associated A β in microglia. Our study using cPLA₂ inhibitors and atomic force microscopy (AFM) shows that inhibiting cPLA₂ activation results in elevated force for membrane tether formation (F_{mtt}), which is a measure for the membrane-cytoskeleton connectivity, and represents a mechanical barrier to endocytic vesicle formation. These data suggest that cPLA₂ facilitates oA β association with microglia in part through the regulation of the cell membrane-cytoskeleton connectivity.

Materials and Methods

Materials.

Human A β ₄₂ was purchased from AnaSpec (Fremont, CA). Hexafluoro-2-propanol (HFIP), dimethyl sulfoxide (DMSO), cOmplete protease inhibitor cocktail, PhosSTOP phosphatase

inhibitor cocktail, β -mercaptoethanol (β -ME), and Triton X-100 were purchased from MilliporeSigma (St. Louis, MO). Ham's F-12 was from Crystalgen Inc. (Commack, NY). Dulbecco's modified eagle medium (DMEM), Penicillin/Streptomycin (P/S), and Dulbecco's Phosphate-buffered Saline (DPBS) were purchased from Life Technologies (Grand Island, NY). Heat-inactivated fetal bovine serum (HI-FBS) and bovine serum albumin (BSA) were from GE Healthcare Life Science (Logan, UT). Methylarachidonyl fluorophosphate (MAFP), pyrrophenone (Pyr), lipopolysaccharide (LPS) and bromoenol lactone (BEL) were purchased from Cayman Chemical (Ann Arbor, MI). Recombinant mouse and rat interferon gamma ($\text{IFN}\gamma$) were from R&D Systems (Minneapolis, MN). Radioimmunoprecipitation assay buffer (RIPA buffer), BCA protein assay kit, ProLongTM diamond antifade mountant with DAPI, SuperSignalTM west femto maximum sensitivity substrate, SuperSignalTM west pico plus chemiluminescent substrate, and RestoreTM PLUS western blot stripping buffer were purchased from Thermo Scientific (Waltham, MA). Paraformaldehyde (PFA), tween[®] 20, and ammonium chloride (NH_4Cl) were purchased from Fisher Scientific (Pittsburgh, PA). Laemmli sample buffer and tris buffered saline (TBS) were from Bio-Rad (Hercules, CA).

Preparation of Oligomeric A β .

Oligomeric A β_{42} (oA β) was prepared according to the protocol as described by Dahlgren et al. 2002 [24].

Primary Rat Microglia Isolation.

All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago. Timed-pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) and primary cortical microglia were isolated as described [25]. A neural tissue dissociation kit (P) from Miltenyi Biotec (Auburn, CA) was used to re-suspend cells [26]. Primary microglia were isolated using CD11b/c microbeads (Miltenyi Biotec) according to the manufacturer's instruction.

Primary Rat Microglial Cell Culture.

Primary rat microglia were seeded onto a 6-well plate after isolation with a density of 5×10^5 cells per well and maintained in DMEM supplemented with 10% HI-FBS and 1% P/S. Cells were treated after two days of culturing.

BV2 Cell Culture.

The immortalized mouse microglia cell line, BV2 cells, were provided by Dr. Rosario Donato (University of Perugia, Italy). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) with 10% HI-FBS and 1% P/S (100 U/ml penicillin and 100 mg/ml streptomycin). At 80-90% confluency, BV2 cells were detached from the culture flasks with a cell scraper and re-seeded ($\sim 10^5$ cells) in new T-75 flasks. Cells were used between passages 15-25.

Measurement of A β association with cells.

Primary rat microglia were cultured in 6-well plates and incubated with serum free DMEM for 1 h before treatments. Cells were pretreated with 10 μ M MAFP or Pyr for 30 min and remained in the medium. Cells were then stimulated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h, followed by incubation with 1 μ M oA β for 1h. BV2 cells were cultured in 35 mm dishes to a confluence of 70-80% and incubated with serum free DMEM for 6 h. Cells were pretreated with 1 μ M, 5 μ M, 10 μ M MAFP or Pyr or 2 μ M BEL for 30 min and remained in the medium thereafter. Cells were then stimulated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1h, followed by incubation with 1 μ M oA β for 15, 30, 45 or 60 min. Cells were washed, lysed and collected using RIPA buffer supplemented with complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail. Total protein concentrations were determined with BCA kits (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. ELISA assay of human A β ₄₂ (Life Technologies) was performed according to the manufacturer's instructions. Data were represented as the total amount of A β divided by the total protein per sample (pg A β / μ g total protein) and normalized by control groups (i.e. Cells treated with oA β alone or LPS + IFN γ + oA β).

Western Blot Analysis.

Cells were cultured in 35 mm dishes at a density of 1×10^5 cells/mL overnight achieve 70-80% confluence. Before treatment, BV2 cells were starved with serum free media for 6h. Cells were stimulated with 1 μ M oA β for 1h, 0.1 or 1 μ g/mL LPS and/or 10 ng/mL IFN γ for various time points to identify the time course for activation of cPLA₂. After treatment, cells were washed twice with cold DPBS and then lysed with 300 μ L cold RIPA buffer supplemented with cOmplete protease and PhosSTOP phosphatase inhibitor cocktails for 15 min at 4 °C. Cell lysates were collected into 1.5 mL Eppendorf tubes by a cell scraper. Cell lysates were then centrifuged at 13,000 rpm for 20 min and supernatants were collected. Western blot analysis of p-cPLA₂ was carried out as previously described [26].

Measurement for the Depletion of Associated A β with Cells by ELISA Assay.

BV2 cells were starved in serum free DMEM for 6 h and pretreated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h or 20 mM NH₄Cl for 30 min. Cells were then treated with 1 μ M oA β for 15 min and replaced with serum-free DMEM. At time 0, the cells were either collected or further incubated for 5, 15, 30 or 60 min in the presence of 1 μ g/ml LPS + 10 ng/ml IFN γ , 10 μ M MAFP, 10 μ M Pyr, 2 μ M BEL or 20 mM NH₄Cl before cell lysis. Total protein and A β quantification analysis was performed in the same manner as the previous section, "Measurement of A β Association with cells".

Immunofluorescence Microscopy of A β at the Cell Surface.

Cells were grown on poly-D-lysine pre-coated coverslips in 12-well plate to a density of 8×10^4 or 5×10^4 cells/well respectively overnight in culture medium. In the next day, cells underwent the aforementioned treatments with LPS, IFN γ , 10 μ M MAFP or Pyr, BEL and/or A β ₄₂. After treatment, cells were washed twice with cold DPBS and fixed with 3.7% PFA in DPBS for 15 min. Cells were washed 3 times with DPBS and then blocked with 5% BSA (w/v) in DPBS for 1 h at room temperature and incubated with Alexa Fluor 488-6E10

(BioLegend, Dedham, MA, 1:200) in 1% BSA in DPBS at 4°C overnight in dark then washed 3 times with DPBS and mounted onto slides with ProLong™ Diamond Antifade Mountant with DAPI and cured at room temperature in dark for 24 h before imaging. Fluorescent images were acquired by a Nikon Eclipse Ti fluorescence microscope with an oil immersion 60X objective lens using a CCD camera. At least 12 images were acquired from each sample. Analysis was performed with CellProfiler software (Carpenter Lab at the Broad Institute of Harvard and MIT) and data was normalized by the cell number.

Mass Conservation for A β ₄₂ in BV2 Cell Culture.

To quantify p-cPLA₂ mediated uptake and processing of A β within microglia, a simple analytical equation was developed based on the principle of mass conservation. The rate of change of intracellular concentration of A β can be defined as

$$\frac{dC_i}{dt} = k_{up}C_o - k_dC_i \quad (1)$$

, where k_{up} is the A β uptake rate constant, k_d the A β depletion rate constant, C_i the intracellular concentration of A β , and C_o the concentration of A β in the medium. In this model, the following assumptions are made: 1) C_o is considered constant, since $C_o \gg C_i$; and 2) k_d is considered to be constant because the depletion of the intracellular A β was not affected by cPLA₂ inhibition via MAFP treatment (Fig. 5b). By defining the relative intracellular A β concentration, $C_i^* = \frac{C_i}{C_o}$, with an initial condition, $C_i^*(t=0) = 0$, solving Eqn.

1 yields:

$$C_i^* = \frac{K_{up}}{K_d} [1 - \exp(-K_d t)] \quad (2)$$

Definitions and units of all parameters in this mass conservation-based model are provided in Table 1. Fitting data from Fig. 4b with Eqn. 2 yields $\frac{k_{up}}{k_d}$ for different concentrations of MAFP (Fig. 6a). Plotting $\frac{k_{up}}{k_d}$ against MAFP concentration exhibits a negative linear relationship between $\frac{k_{up}}{k_d}$ and MAFP concentration (Fig. 6b). Since Fig. 5b suggested k_d was not changed by MAFP, k_{up} decreased linearly with increasing dose of MAFP.

Membrane Tethering Force Measurement.

BV2 cells were cultured on poly-d-lysine pre-coated coverslips. Cells were pretreated with 10 μ M MAFP or without for 30 min and then treated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h. Then the coverslip was washed with PBS and mounted onto a glass slide with Epoxy (3M, St. Paul, MN), cells were kept wet during all the procedures. Tethering forces were measured using a MFP-3D Bio atomic force microscopy (AFM) (Oxford Instruments

Asylum Research, Santa Barbara, CA). TR400PSA cantilevers (also known as OTR4, Oxford Instruments Asylum Research) were used. Spring constant of the AFM tips were range from 82.69 to 100.24 pN/nm. Indentation for each measurement is 1 μ m with a rate of 1 Hz and the force curve for each measurement was recorded by the software (AR14, Oxford Instruments Asylum Research). At least 10 cells and 5 force curves per cell were analyzed for each group.

Statistical Analysis.

Data is presented as the mean $\pm$ standard deviation (SD) or standard error (SEM) from at least three independent experiments. Statistical analysis was carried out with one-way ANOVA and Bonferroni post hoc tests in GraphPad Prism (Version 6.01). P-values less than 0.05 are considered statistically significant.

Results

cPLA₂ Inhibitors Decrease A β Association with Primary Rat Microglia.

As compared to the monomeric and fibrillar forms of A β , oA β has been reported to be the most neurotoxic form of aggregates [24]. In this study, we focus on oA β association with microglia. oA β was prepared as previously described [24] and was characterized using AFM for quality control as previously described [27]. To demonstrate the role of cPLA₂ in the association of oA β with microglia, primary rat microglia were pretreated with cPLA₂ inhibitors, MAFP and Pyr, followed by treatment with 1 μ M oA β for 1h. A β association with cells was assessed using ELISA. Pre-stimulation of cells with LPS + IFN γ , known to activate cPLA₂, did not increase A β association with cells (Fig. 1a). Interestingly, both Pyr and MAFP decreased A β association with primary rat microglia (Fig. 1a), suggesting a role for cPLA₂ in A β association with microglia. However, when we performed fluorescent immunostaining of A β without cell permeabilization (i.e. fluorescently labeling A β at the cell surface only), both cPLA₂ inhibitors did not affect A β association with primary rat microglia at the cell surface (Fig. 1b and c). These results suggest that cPLA₂ inhibitors decrease oA β internalization in primary microglia.

Activation of cPLA₂ in BV2 cells by oA β and LPS + IFN γ .

To further study the roles of cPLA₂ in A β association with microglia, we utilized immortalized mouse microglia (BV2 cells). BV2 cells are the most frequently used cell lines in studies of microglia and have been shown to demonstrate similar inflammatory responses and A β accumulation to primary microglia [28,29,26]. Here we demonstrate the activation of cPLA₂ in BV2 cells. When BV2 cells were exposed to 1 μ M oA β for 1h, activation of cPLA₂ was reflected by the increase in phosphorylated cPLA₂ (p-cPLA₂) (Fig. 2a). cPLA₂ was also activated when cells were exposed to 1 μ g/mL LPS + 10ng/mL IFN γ and 1 γ g/mL LPS for 1 h (Fig. 2b). When cells were exposed to 1 γ g/mL LPS + 10ng/mL IFN γ , the increase in p-cPLA₂ was activated in a time-dependent manner with a maximal activation after 2h (Fig. 2c). p-cPLA₂ was suppressed when cells were pretreated with MAFP for 30 min (Fig. 2d). Based on these results, BV2 cells were stimulated with 1 γ g/mL LPS + 10ng/mL IFN γ for 1 h, and different concentrations (5 and 10 μ M) of MAFP were applied to further study the role of cPLA₂ in oA β association with microglia.

cPLA₂ Inhibitors Decreases A β Association with BV2 cells.

Consistent with the results using primary rat microglia (Fig. 1a), we found that pre-stimulation of BV2 cells with LPS + IFN γ did not increase A β association with cells (Fig. 3a). Inhibition of cPLA₂ with MAFP and Pyr decreased A β association with BV2 cells (Fig. 3b and d). MAFP and Pyr did not affect A β association with BV2 cells at the cell surface (Fig. 4a and b). These results suggest that inhibition of cPLA₂ by MAFP and Pyr reduced A β association with microglia, and that mainly occurred intracellularly. Since MAFP at high concentrations can also inhibit calcium-independent PLA₂ (iPLA₂), we found that BEL, a specific iPLA₂ inhibitor, also had no effect on A β association with cells (Fig. 3c), thus ruling out the involvement of iPLA₂.

Fig. 3a also suggests that intracellular A β content had already reached equilibrium after exposing cells to oA β for 15 min. However, A β association with cells was decreased by MAFP and Pyr in a dose-dependent manner (Fig. 3b and d). These results suggest that an equilibrium of associated A β was reached within 15 min, when the rate of oA β uptake equaled the rate of A β depletion in cells. Inhibition of cPLA₂ by MAFP and Pyr decreased A β association with cells, indicating a shift of this equilibrium.

Inhibition of cPLA₂ does not Affect Depletion of Associated A β in BV2 Cells.

A β association with cells is governed by both the rates of oA β uptake and depletion of associated A β . Although the rate of oA β uptake cannot be measured directly, it can be indirectly evaluated by measuring both oA β association with cells (Fig. 3) and the depletion of associated A β in cells (Fig. 5). To study the depletion of associated A β in BV2 cells, cells stimulated by LPS + IFN γ were exposed to 1 μ M oA β for 15 min, followed by replacing oA β -containing media with standard serum-free media. Cells were then incubated in standard serum-free media for 5, 15, 30 and 60 min and lysed to quantify associated A β in cells using ELISA. Fig. 5a shows that pre-stimulation of cells with LPS + IFN γ did not affect depletion of associated A β . Associated A β decreased and leveled off at ~35% within 5 min and remained unchanged even at the 60-min time point (Fig. 5a). The depletion of associated A β can be the result of A β recycling back to the media and/or hydrolysis of A β in lysosomes. To examine the cause of associated A β depletion, we treated cells with 20 mM NH₄Cl, an inhibitor of lysosomal function [30-32] after the treatment of cells with oA β . We found that the depletion of associated A β was completely abrogated in cells pretreated with NH₄Cl (Fig. 5a). These results indicate that degradation of associated A β in lysosomes is the major cause of the depletion of associated A β in cells. MAFP, Pyr and BEL did not impose any effects on the depletion of associated A β (Fig. 5b), indicating that neither cPLA₂ nor iPLA₂ plays a role in the degradation of associated A β .

Inhibition of cPLA₂ Decreases the oA β Uptake Rate Constant.

Since cPLA₂ did not affect the rate of associated A β depletion in cells (Fig. 5b), data depicting the effects of cPLA₂ inhibition on A β association with cells in Fig. 3b suggest that oA β uptake rate constant decreases with increasing dose of MAFP. By applying the principle of conservation of mass (see detailed derivation in the Methods section), the normalized concentration of associated A β in cells, C_i^* , at different time points can be fit

with a mathematical model, $C_i^* = \frac{k_{up}}{k_d} \left(1 - \exp \left(-k_d \cdot t \right) \right)$, to yield the ratio of the oA β uptake rate constant to the A β depletion rate constant, $\frac{k_{up}}{k_d}$, for different doses of MAFP (Fig. 6a).

Plotting $\frac{k_{up}}{k_d}$ against the concentration of MAFP, showing that $\frac{k_{up}}{k_d}$ decreased linearly with an increasing dose of MAFP (Fig. 6b). Since Fig. 5b suggests that k_d was independent of cPLA₂ activation (i.e. k_d remained unchanged with different dose of MAFP), Fig. 6b suggests that the oA β uptake rate constant, k_{up} , decreased linearly with an increasing dose of MAFP.

Inhibition of cPLA₂ Increases the Membrane-Cytoskeleton Connectivity.

Membrane-cytoskeleton connectivity governs many mechanochemical processes [23], including endocytosis [23,33,34]. Membrane-cytoskeleton connectivity can be quantitatively evaluated by the measurement of force for membrane tether formation (F_{mtf}) using laser tweezers [33,35,36] and atomic force microscopy (AFM) [37,38]. Here, we employed AFM to study the mechanical process underlying the effects of cPLA₂ inhibition on oA β association with BV2 cells. Fig. 7a is a typical force curve obtained from AFM measurement. The red curve represented the AFM cantilever approaching to the cell in three steps: (1) the cantilever was approaching to the cell without contact from A to B; (2) the cantilever contacted the cell surface at B; (3) the cantilever moved further to make an indentation from B to C (Fig. 7a). The red curve from B to C, where cell indentation was made, is typically fitted with the Hertz model to estimate cell stiffness, which is not the focus of this study. The blue curve represented the cantilever retracting from the cell (Fig. 7a). Along the retraction curve, a sudden release of force was observed at point F, where the rupture of a membrane tether occurred (Fig. 7a). The step change in the force curve during the rupture of a membrane tether was used to measure F_{mtf} . Fig. 7b showed that MAFP increased F_{mtf} in unstimulated cells and in cells stimulated with LPS + IFN γ , indicating that inhibition of cPLA₂ activation results in increased membrane-cytoskeleton connectivity in cells. These results suggest that activated cPLA₂ helps attenuate the increase in the membrane-cytoskeleton connectivity to maintain endocytosis of oA β in stimulated microglia.

Discussion

Microglia have been found to be immunoreactive for cPLA₂ in central nervous system (CNS) injury and neurodegenerative diseases, including Alzheimer's disease [2]. Upregulation of cPLA₂ in microglia can be induced through the redox-sensitive NF- κ B activation, and in turn, cPLA₂ plays a major role in A β -induced NADPH oxidase activity, superoxide production, prostaglandin E₂ (PGE₂) formation, iNOS expression, and NO production in microglia [39]. However, AD-related cPLA₂ function has yet to be fully elucidated. Our data clearly show that cPLA₂ plays a role in oA β association with microglia through regulation of the membrane-cytoskeleton connectivity.

The soluble, monomeric, and oligomeric forms of A β aggregate to produce fibrillar A β (fA β). However, microglia do not effectively clear fA β [40,41], and oA β has been found to be the most neurotoxic among other forms of A β [24]. Therefore, understanding the mechanism(s) involving in the maintenance of oA β homeostasis provides important information regarding the development of AD pathology. Although receptor-mediated phagocytosis of fA β has been studied extensively, the mechanism(s) underlying oA β association with microglia are largely unknown. It has been reported that microglia mediate the clearance of soluble A β through fluid phase macropinocytosis [42]. The uptake of oA β is dependent on both actin and tubulin dynamics, but does not involve clathrin assembly, coated vesicles or membrane cholesterol, and the uptake of soluble A β and fA β occurs largely through distinct mechanisms and upon accumulation are segregated into separate subcellular vesicular compartments [42,43]. These previous findings suggest that oA β uptake by microglia is intimately related to the composition and physical properties of the plasma membrane, and the interaction between the plasma membrane and cytoskeleton beneath the plasma membrane. Since cPLA₂ is a lipid-modifying enzyme, and increased activities have been found in AD brains, it is important to investigate the role of cPLA₂ in oA β association with microglia.

Lipid-modifying enzymes induce membrane curvature for biogenesis of transport carriers [44,45]. As activated cPLA₂ targets cellular membranes to hydrolyze phospholipids to produce fatty acids and lysophospholipids, it has been hypothesized that converting cylinder-shaped phospholipids into wedge-like lysophospholipids by activated cPLA₂ alters lipid geometry favorable for the generation of positive membrane curvature, and supports the budding of tubular transport [46,47]. In turn, cPLA₂ was found to be a key driver for recycling through the clathrin-independent endocytic route [48]. These previous findings suggest that activated cPLA₂ facilitates clathrin-independent fluid phase macropinocytosis of oA β in microglia.

In addition to membrane bending or curvature, the mechanochemical process of endocytosis, including receptor-mediated and fluid phase endocytosis, is controlled not only biochemically through interaction with regulatory proteins, but also physically through an apparently continuous adhesion (or connectivity) between plasma membrane lipids and cytoskeletal proteins [23]. Membrane-cytoskeleton adhesion is the key mechanical resistance for the formation of endocytic vesicles, and has been found to be the major factor in the force for membrane tether formation (F_{mtf}) [33]. Therefore, F_{mtf} can be a measure for the membrane-cytoskeleton connectivity. Modifying the plasma membrane by exposing cells to amphiphilic compounds including detergents, lipids, and solvents, has been demonstrated to cause a parallel decrease in F_{mtf} and rise in endocytosis rate [35,49]. Here we employed atomic force microscopy (AFM) to measure F_{mtf} for studying the mechanical mechanism underlying the effects of cPLA₂, a lipid-modifying enzyme, on oA β association with microglia. F_{mtf} of (LPS+IFN γ)-stimulated cells was higher than that of control cells (Fig. 7b). This result is consistent with the notion that the inflammatory environment in the AD brain may increase membrane-cytoskeleton connectivity to lower the ability of microglia to macropinocytose oA β from the milieu, acting to promote disease pathogenesis. In fact, anti-inflammatory agents have been reported to enhance the ability of microglia to process A β [50-53], and prevent neuroinflammation, lower A β levels and improve cognitive

performance in Tg APP mice [54]. Interestingly, inhibition of cPLA₂ by MAFP further increased F_{mtf} both unstimulated and stimulated cells (Fig. 7b). These results suggest that stimulated cells have higher membrane-cytoskeleton connectivity, and cPLA₂ activation attenuated the increase in membrane-cytoskeleton connectivity to help maintain the endocytosis rate.

Although cPLA₂ did not play a role in the degradation of associated A β (Fig. 5b), our data clearly show that ~ 65% of associated A β was rapidly digested through lysosomal activities (Fig. 5a). ~35% of associated A β remained in the cells and suggested that there was no significant A β recycling back to culture medium (Fig. 5a). These results are consistent with previous findings that soluble A β is rapidly trafficked into late endolysosomal compartments and subject to degradation without significant A β recycling back to culture medium [42]. Once accumulated, A β microaggregates colocalize to cellular fractions containing the lysosomal markers β -hexosaminidase and acid phosphatase [55].

cPLA₂ has been implicated in neurodegenerative diseases and brain injury including AD and cerebral ischemia [56-58,3]. Therefore, examining the effects of cPLA₂ activities in brains using animal models have been a research focus. For example, genetic ablation or reduction of cPLA₂ protected human amyloid precursor protein (hAPP) mice against A β -dependent deficits in learning and memory, behavioral alterations and premature mortality [59]. Lithium activated brain phospholipase A₂ and improves memory in rats [60]. *In vivo* inhibition of cPLA₂ in rat brain decreased the levels of total Tau protein [61]. Another line of research on cPLA₂ is to address its functional roles in AD pathology. Modulations of cPLA₂ activation by A β stimulation and inhibitors altered membrane fluidity and molecular order [62,19,63]. Therefore, A β -activated cPLA₂ may also alter functions of organelles through its ability to alter cellular membrane properties. For example, cPLA₂ has been found to mediate A β -induced mitochondrial dysfunction in astrocytes [64]. Consistently, inhibition of cPLA₂ diminished A β -induced neurotoxicity [59]. Both NMDA and oA β activated cPLA₂ leading to release of arachidonic acid (AA) in primary rat neurons [20]. In turn, AA served not only as a precursor for eicosanoids, but also as a retrograde messenger which plays a role in modulating synaptic plasticity [20]. A β also induced elevation of APP protein expression mediated by cPLA₂, PGE₂ release, and cAMP response element binding protein (CREB) activation via protein kinase A pathway in primary rat cortical neuronal cultures [65]. In this study, our results contribute to the functional roles of cPLA₂ in AD pathology by demonstrating its role in facilitating oA β association with microglia through its regulation of membrane-cytoskeleton connectivity. Better understanding the functional roles of cPLA₂ in AD pathology should provide critical insights into the development of therapeutic strategies for AD treatment.

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Fig. 1.

A β association with primary rat microglia. (a) Quantification of A β association with primary rat microglia using ELISA assay. Cells were pretreated with or without 10 μ M Pyr or MAFP for 30 min, followed by treatment with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h, and then cells were exposed to 1 μ M oA β for 1 h. Data are represented as the ratio of A β to total protein, and normalized by the control group (oA β group). Data are shown as mean $\pm$ SD from at least 3 independent experiments (n = 3). *** P < 0.001 comparing with the LPS + IFN γ + oA β group. (b) Representative immunofluorescent images of A β association with primary rat microglia at the cell surface. A β was stained with Alexa Fluor 488-6E10 antibody

without cell permeabilization. (c) Quantification of immunofluorescent images of A β association with primary rat microglia at the cell surface. MAFP and Pyr did not impose any effect on A β association with primary rat microglial cell surface.

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Fig. 2.

CPLA₂ activation in BV2 cells. Cells were treated with (a) 1 μM oAβ₄₂ for 1 h, (b) LPS and/or IFNγ (10 ng/ml in all cases except control) for 1 h and (c) 1 μg/mL LPS and 10 ng/mL IFNγ from 0-4 h. (d) BV2 cells were pretreated with 0, 1, 5 or 10 μM MAFP for 30 min, then all groups were treated with LPS (1 μg/ml) + IFNγ (10 ng/ml) for 1 h. The ratio of p-cPLA₂/cPLA₂ is represented as the fraction percentage of the control group. Data are shown as mean ± SD from 3-4 independent experiments (n = 3 or 4), at least 6 independent experiments for (d) (n = 6). *P < 0.05, **P < 0.01, ***p < 0.001, compared with the control group; # P < 0.05, ### P < 0.001 compared with the LPS + IFNγ group in (d).

Fig. 3.

Effect of CPLA₂ inhibition on Aβ association with BV2 cells. (a) Quantification of associated Aβ in BV2 cells pre-treated with LPS + IFNγ using ELISA assay. Cells were treated with or without LPS (1 μg/ml) + IFNγ (10 ng/ml) for 1 h, followed by 1 μM oAβ₄₂ treatment for 15, 30, 45 and 60 min. Data are represented as the ratio of Aβ₄₂ to total protein, and normalized by the oAβ group at 15 min. Data are shown as mean ± SD from 3 independent experiments (n = 3). No significant difference was observed between these two groups at any time point. (b) Quantification of associated Aβ in BV2 cells pre-treated with MAFP using ELISA assay. Cells were pretreated without or with MAFP (1 μM, 5 μM and 10 μM) for 30 min, followed by 1 μg/ml LPS +10 ng/ml IFNγ treatment for 1 h, then cells were incubated with 1 μM oAβ for 15, 30, 45 and 60 min. Data are represented as the ratio of Aβ to total protein, and normalized by the LPS + IFNγ + oAβ group at 15 min. Data are shown as mean ± SD from 3 independent experiments (n = 3). **P < 0.01, ***P < 0.001 compared with the LPS + IFNγ + oAβ group at the corresponding time. (c) Quantification of associated Aβ in BV2 cells pre-treated with BEL using ELISA assay. Cells were pretreated without or with 2 μM BEL for 30 min, followed by 1 μg/ml LPS +10 ng/ml IFNγ treatment for 1 h, then cells were incubated with 1 μM oAβ for 15, 30, 45 and 60 min. Data is represented as the ratio of Aβ to total protein, and normalized by the LPS + IFNγ + oAβ group at 15 min. Data are shown as mean ± SD from 3 independent experiments (n = 3). **P < 0.01 compared with the LPS + IFNγ + oAβ group at the corresponding time. (d)

Quantification of associated A β in BV2 cells pre-treated with Pyr using ELISA assay. Cells were pretreated without or with Pyr (1 μ M, 5 μ M and 10 μ M) for 30 min, followed by 1 μ g/ml LPS + 10 ng/ml IFN γ treatment for 1 h, then cells were incubated with 1 μ M oA β for 15 and 60 min. Data is represented as the ratio of A β to total protein, and normalized by the LPS + IFN γ + oA β group at 15 min. Data are shown as mean $\pm$ SD from 3 independent experiments (n = 3). *P<0.05, **P < 0.01, ***P < 0.001 compared with the LPS + IFN γ + oA β group at the corresponding time.

Fig. 4.

A β association with BV2 cells surface. Cells were pretreated with or without 10 μ M MAFP or Pyr or 2 μ M BEL for 30 min and treated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h, followed by incubation with 1 μ M oA β for 15 min. Fluorescent intensities per cell were normalized by the oA β group. Data are shown as mean $\pm$ SD from 3 independent experiments ($n = 3$) (at least 12 images were analyzed for each group per experiment). * $P < 0.05$ compared with the LPS + IFN γ + oA β group. (a) Representative immunofluorescent images of A β association with BV2 cell surface. A β was stained with Alexa Fluor 488-6E10 antibody without cell permeabilization. (b) Quantification of immunofluorescent images of A β association with BV2 cell surface. MAFP and Pyr did not impose any effect on A β association with BV2 cell surface.

Fig. 5.

Depletion of associated A β in BV2 cells. (a) Quantification of associated A β in BV2 cells treated with NH₄Cl using ELISA assay. BV2 cells were treated with 1 μ g/ml LPS + 10 ng/ml IFN γ for 1 h or 20 mM NH₄Cl for 30 min, followed by adding 1 μ M oA β for 15 min. Afterwards the media was replaced with 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 after incubating cells with 1 μ M oA β for 15 min. Data are represented as the ratios of A β to total protein, and normalized by the oA β group at 0 min. Data are shown as mean $\pm$ SD from at least 3 independent experiments (n = 3). ***P<0.001 compared with the oA β group at 0 min; # P < 0.05, ## P < 0.01 compared with the LPS + IFN γ + oA β group. (b) Quantification of associated A β in BV2 cells treated with MAFP, BEL or Pyr using ELISA assay. BV2 cells were treated with 1 μ g/ml LPS + 10 ng/mL IFN γ for 1 h, followed by 1 μ M oA β incubation for 15 min. Afterwards, cells were treated with serum-free media, 10 μ M MAFP, 10 μ M Pyr or 2 μ M BEL for 5, 15, 30 and 60 min before cell lysis. Data are represented as the ratio of A β ₄₂ to total protein, and normalized by the LPS + IFN γ + oA β group at 0 min. Data are shown as mean $\pm$ SD from at least 3 independent experiments (n = 3).

Fig. 6.

Application of the mass conservation model to the experimental ELISA Aβ association with BV2 cells data. (a) Normalized concentration of Aβ in cells, C_i^* , from Fig. 4B were plotted against time and fit with a mathematical model, $C_i^* = \frac{k_{up}}{k_d} \left(1 - \exp(-k_d \cdot t) \right)$, to yield the ratio of the oAβ uptake rate constant to the Aβ depletion rate constant (k_{up}/k_d) for different doses of MAFP. (b) The ratio of the oAβ uptake rate constant to Aβ depletion rate constant (k_{up}/k_d) linearly decreased with increasing dose of MAFP. Data are represented as the mean $\pm$ SEM.

Fig. 7.

Membrane tethering force of BV2 cells. (a) A typical AFM force curve from the cell. Red line is the approaching curve and blue line is the retraction curve. Magnified the retraction curve at point F shows a sudden release of force as a membrane tethering force where a membrane tethering rupture event happened. The membrane tethering force measured from this event is around 50 pN. (b) Membrane tethering force in BV2 cells. Cells were pretreated with or without 10 μ M MAFP for 30 min, followed by 1 μ g/ml LPS + 10 ng/ml IFN γ treatment for 1 h. Data are represented as the mean $\pm$ SEM from 45-128 membrane tethering events ($n = 45 - 128$). *** $P < 0.001$ compared with the control group; # $P < 0.05$ compared with the LPS + IFN γ group.

Table. 1

Defining the variables of the mass conservation-based model.

Variable	Definition	Units
C_i	Concentration of A β associated in the cell	pg A β /μg total protein
C_o	Concentration of oA β in medium	μM oA β
C_i^*	The ratio of A β in the cell to oA β in medium (C_i/C_o)	(pg A β /μg total protein)/(μM oA β)
k_{up}	oA β uptake rate constant	(pg oA β /μg total protein)/(μM A β)(min)
k_d	A β depletion rate constant	(min ⁻¹)
$k_{up}C_o$	Rate of oA β uptake	(pg oA β /μg total protein)/(min)
k_dC_i	Rate of A β depletion	(pg A β /μg total protein)/(min)
dC_i/dt	Rate of change of associated A β concentration in the cell	(pg A β /μg total protein)/(min)
t	Time	min