Lysophosphatidylcholine increases the neurotoxicity of Alzheimer's amyloid $\beta_{1\text{-}42}$ peptide: Role of oligomer formation

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Running title: LPC increases $A\beta_{1\text{-}42}$-induced neurotoxicity

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Abstract

Oligomer formation is considered as a critical process for the neurotoxic effects of Alzheimer's amyloid β (Aβ) peptide. Previously we have demonstrated that lysophosphatidylcholine (LPC) increases the oligomer formation of $A\beta_{1-42}$, the major Aβ peptide found Alzheimer's disease (AD) lesions. In this study, we have investigated whether LPC affects the neurotoxic effects of $A\beta_{1-42}$ in a neuronal cell line (A1) culture. MTT assay revealed that up to 10 μM concentration, LPC did not affect A1 cell viability. $A\beta_{1-42}$ decreased the cell viability, and such effect was dose dependently enhanced by LPC. However, neither LPC nor $A\beta_{1-42}$, alone or in combination increased LDH release from A1 cells after 24 h treatment. TUNEL assay showed that LPC increased $A\beta_{1-42}$-induced apoptotic cell number. To determine the underlying mechanisms, the proteins implicated in apoptosis pathways including Bcl-2- and caspase-family were analyzed by Western blotting. The results demonstrated that $A\beta_{1-42}$ decreased Bcl-2 in A1 cells at 24 h, whereas LPC had no effect at any time point. Both LPC and $A\beta_{1-42}$ increased Bax level at 24 h, and their combined stimulation showed a synergistic effect. Similar synergistic effect of LPC and $A\beta_{1-42}$ on caspase9 activation was observed. Dot blot immunoassay and Western blotting showed that LPC augmented $A\beta_{1-42}$ oligomer formation in cell culture medium. Removing LPC-induced early formed $A\beta_{1-42}$ oligomer from the culture medium by immunoprecipitation decreased active caspase9 level and neurotoxicity, as revealed by Western blotting and MTT assay. Furthermore, DHE assay showed that $A\beta_{1-42}$ increased reactive oxygen species level in A1 cells, such effect was further enhanced by LPC. Thus, our results demonstrated

that LPC increased the oligomer formation process of Aβ_{1-42} peptide in culture condition, and consequently increased apoptotic neuronal death. Such process might be important for the pathogenesis of AD, and inhibition of LPC generation could be a therapeutic target for the disease.

Key words: Lysophosphatidylcholine, Amyloid β, Alzheimer’s disease, oligomer, neuronal apoptosis.

1 **Introduction**

2 Alzheimer's disease (AD) is a common neurodegenerative disorder,
3 manifested clinically as progressive dementia (Rowland et al., 2010).
4 Histopathological examination of AD brains demonstrated the presence of Congo
5 red positive areas of amyloid deposits, referred as AD plaques, in the
6 hippocampus and cortical regions (Navarro et al., 2013). The deposited amyloid
7 is mainly composed of amyloid β (Aβ), a 39 to 42 amino acids long peptide
8 derived from transmembrane amyloid precursor protein (APP) (Barrow and
9 Zagorski, 1991; Selkoe, 1996). In addition to amyloid deposit, dystrophic neurons
10 and reactive glia are found in typical AD plaques (Selkoe, 1991).

11 Several animal and genetic studies are suggesting that increased
12 production Aβ peptide is one of the main causes of AD (Campion et al., 1995;
13 Goate et al., 1991; Price and Sisodia, 1998; Richards et al., 1991; Xia et al.,
14 1997; Yoshioka et al., 1991). After deposition in the affected areas, this peptide is
15 believed to induce a degenerative process leading to loss of neurons. Aβ peptide
16 is deposited mainly as aggregated form, which has been shown to be intimately
17 associated with dystrophic neurons and reactive glial cells (Selkoe, 1991). In vitro
18 cell culture studies confirmed the neurodegenerative properties of Aβ peptides;
19 and the aggregated form of the peptide, especially the oligomers are proved to
20 be more toxic than that of monomeric species (Lorenzo and Yankner, 1994;
21 Malaplate-Armand et al., 2006). Consequently, it is suggested that increased
22 production as well as aggregation of the peptide play an important role in the
23 neurodegenerative process. In vitro fibril formation studies showed that at least
24 micromolar concentration of the peptide is required to induce fibril formation

(Sheikh and Nagai, 2011). However, in AD condition, the concentration of the peptide in the brain tissue is at nanomolar level (Haugabook et al., 2001; Pacheco-Quinto et al., 2006). Therefore, it is conceivable that some unknown biomolecule(s) might influence the in vivo microenvironment, which leads to aggregation of the peptide at such a low concentration (Yanagisawa et al., 1995). Identification of such biomolecule(s) might provide a better understanding of Aβ-induced neurodegeneration in AD.

As an essential component of cell membrane, phospholipids metabolism is intimately associated with various types of cellular functions (Kent et al., 1991). A substantial number of reports have suggested the alteration of phospholipid metabolism in AD (McLaurin and Chakrabartty, 1997; Michikawa et al., 2001; Sun et al., 2012). For example, the activity of phospholipase A2, an enzyme that generates lysophosphatidylcholine (LPC) from phosphatidylcholine (PC), is increased in AD brains (Sun et al., 2012). Accordingly, the concentration of LPC is likely to change in such condition. Indeed, a report showed that the concentration of LPC is increased in the white matter of aged human brains exhibiting senile atrophy of the Alzheimer type (Wender et al., 1988). Moreover, the LPC to PC ratio is decreased in the CSF of AD patients (Mulder et al., 2003). Hence, alteration of LPC metabolism might have an important role in the pathophysiology in AD.

Recently we have demonstrated that LPC affects the aggregation process of $A\beta_{1-42}$ peptide. It decreases critical micellar concentration (CMC) of the peptide resulting aggregation at a lower concentration, and also progresses the aggregation at a faster rate (Sheikh and Nagai, 2011). As a result, the

1 oligomers of the peptide appear at an earlier time point. Because of high
2 neurotoxic effect of Aβ oligomers (Malaplate-Armand et al., 2006), we
3 hypothesized that LPC might enhance Aβ-induced neurotoxicity. To test the
4 hypothesis, we have investigated about the role of LPC on Aβ-induced
5 neurodegeneration, and elucidated the underlying mechanisms using an in vitro
6 neuronal cell culture system. We have found that LPC increased Aβ-induced
7 neurotoxicity by enhancing the oligomer formation of the peptide.
8

1 **Materials and Methods**

2 **Materials**

3 $A\beta_{1-42}$ peptide was purchased from Peptide Institute (Osaka, Japan). In
4 cold condition, the peptide was dissolved in 0.1% NH_3 at a concentration of 250
5 μM, aliquoted and stored at -70°C until use. Chromatographic data provided by
6 the manufacturer demonstrated the monomeric purity of the peptides. LPC was
7 obtained from Avanti Polar Lipids (Alabaster, AL). DMEM medium and cell
8 culture grade PBS were purchased from Wako Pure Chemicals (Richmond,
9 VA), and FBS were from Gibco (Invitrogen, Carlsbad, CA). Prestained protein
10 size marker for Western blotting was purchased from NIPPON Genetics
11 EUROPE GmbH (Duren, Germany) and 4-20% Tris-glycine polyacrylamide gel
12 was from Bio-Rad (Hercules, CA, USA). Nitrocellulose membrane for dot blot
13 assay was obtained from Millipore (Billerica, MA).

14 **Cell culture**

15 A human neuronal cell line (A1) was generated by somatic fusion of a
16 human primary fetal cerebral neuron and a human neuroblastoma cell (Nagai et
17 al., 2002). A1 cells showed similar morphological, electrophysiological and
18 expressional features like primary neurons in culture. A1 cells were cultured in
19 5% FBS containing DMEM medium. For differentiation, 20 μM of retinoic acid
20 was used for 48 h. During stimulation with $A\beta_{1-42}$ and LPC, the concentration of
21 FBS was reduced to 1%. The photomicrographs of the cultured cells were
22 obtained with an inverted cell culture microscope (Olympus CK-2, Olympus,
23 Tokyo, Japan) equipped with a digital photograph acquiring system (Olympus).

24 **MTT cell viability assay**

1 The effect of LPC on $A\beta_{1-42}$-induced neuronal toxicity was evaluated by
2 MTT cell viability assay, as described previously (Nagai et al., 2005). Briefly, A1
3 cells ($3X10^3$/well) were seeded on the wells of a 96-well cell culture plate and
4 cultured for 24 h. Then neuronal differentiation was done by RA treatment for 48
5 h. The cells were treated with indicated concentrations of LPC, $A\beta_{1-42}$ or
6 combined $A\beta_{1-42}$ and LPC in 100 µl of 1% FBS containing DMEM for indicated
7 time. After treatment, 20 µl of MTT solution (Dojindo Molecular Technologies,
8 Rockville, MD) (5 mg/ml) was added to the culture medium and incubated for
9 3.5 h at 37°C. Then the medium was removed carefully, MTT solvent (4 mM
10 HCl, 0.1% Nondet P-40 in isopropanol) was added and further incubated for 15
11 min at room temperature after protecting from light. Then the absorbance was
12 read at 590 nm. The absorbance of the cells of normal culture condition was
13 used as a control.

14 **Determination of Lactate Dehydrogenase (LDH) level in the culture**
15 **supernatant**

16 The effect of LPC on $A\beta_{1-42}$-induced release of LDH into the culture
17 supernatant was evaluated using a LDH cytotoxicity assay kit (Cayman
18 Chemical Company, Ann Arbor, MI), following the manufacturer's instruction.
19 Briefly, A1 cells ($3X10^3$/well) were seeded on the wells of a 96-well cell culture
20 plate and cultured for 24 h. Then neuronal differentiation was done by RA
21 treatment for 48 h. The cells were treated with indicated concentrations of LPC,
22 $A\beta_{1-42}$ or combined $A\beta_{1-42}$ and LPC in 125 µl of 1% FBS containing DMEM for
23 indicated time. After treatment, the plate was centrifuge at 400 x g for 5 min,
24 and 100 µl of culture supernatant was transferred to another 96-well plate. Then

100 μl of LDH reaction solution, supplied by the manufacturer, was added to the culture supernatant, incubated at room temperature for 30 min on an orbital shaker, and the absorbance was read at 490 nm with a plate reader. The absorbance of 1% FBS containing DMEM was subtracted from values of culture supernatant samples. A standard curve was made using LDH standard supplied by the manufacturer, and the level of LDH in a sample was measured using that standard curve.

Terminal deoxynucleotidyl transferase dUTP-biotin nick-end-labeling (TUNEL) assay

The TUNEL assay was performed using a kit (In Situ Cell Death Detection Kit, POD, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, A1 cells were cultured on a glass chamber slide and differentiated. After receiving appropriate treatment, the cells were washed with PBS, fixed with 4% Paraformaldehyde in PBS (pH 7.4) and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate. Then the apoptotic cells were detected by labeling the DNA nicks with fleurescein conjugated nucleotides using label solution and enzyme solution provided by the manufacturer. For counting total cells, nuclei were stained with Hoechst. The cells were then examined under a fluorescent microscope (NIKON, ECLIPSE E600), counted in 5 randomly selected high power fields, and the average was considered as representative number of apoptotic cells in a condition.

Dot-blot immunoassay

After incubation at 37°C in 1% FBS containing DMEM for indicated time,

2μg of $A\beta_{1-42}$ peptide equivalent sample was applied to a nitrocellulose membrane using a manifold. Then the membrane was immunoblotted with an oligomer-specific antibody (A11, rabbit polyclonal, Invitrogen). This oligomer-specific antibody is reported to react specifically with a variety of soluble oligomeric species of proteins or peptides regardless of their amino acid sequence, and does not react with either monomer species or insoluble fibrils (Kayed et al., 2003). The antibody-oligomer immunoreaction was detected by infrared dye-conjugated anti-rabbit IgG and Odyssey infrared dye scanning system (Li-Cor, Lincoln, NE, USA), according to the manufacturer's instructions.

Western blot analysis

Total protein was isolated from cultured A1 cells after appropriate treatment using ice cold RIPA buffer (PBS, pH 7.4, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1%SDS, 10 mg/ml PMSF, and 1 mg/ml aprotinin). Forty micrograms of total protein was separated by 10% or 4-20% gradient SDS polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Millipore, Billerica, MA). Then the membrane was immunoblotted with anti-Bax (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (rabbit polyclonal, Santa Cruz), anti-caspase8 (rabbit polyclonal, Sigma,St Louis, MO) or anti-caspase9 (mouse monoclonal, Santa Cruz) antibodies. Immunoreactive proteins were detected using infrared dye-conjugated anti-rabbit or anti-mouse IgG and Odyssey infrared dye scanning system (Li-Cor), according to the manufacturer's instructions. Equal loading of cell lysate was verified by β-Actin immunoblotting.

1 For determination of Aβ oligomers in DMEM medium, the samples were
2 concentrated using a vacuum evaporator, 2 μg peptide equivalent samples were
3 separated by 4-20% gradient SDS polyacrylamide gel electrophoresis in non-
4 reducing condition and transferred to PVDF membranes (Millipore). Then the
5 membrane was immunoblotted with anti-Aβ antibody (mouse monoclonal, Santa
6 Cruz). Immunoreactive protein was detected using infrared dye-conjugated anti-
7 mouse IgG and Odyssey infrared dye scanning system (Li-Cor)

8 **Immunoprecipitation and preparation of conditioned media**

9 $A\beta_{1-42}$ (10 μM) was added to DMEM medium with or without LPC (10
10 μM) and incubated at 37°C for 8 h without agitation. Then the oligomers were
11 immunoprecipitated using an oligomers specific antibody (A11, Invitrogen) and
12 Protein G agarose. Immunoprecipitate was pelleted by centrifugation, the
13 supernatant medium was collected and 1% FBS (Gibco) was added. The media
14 without immunoprecipitation or immunoprecipitation with normal rabbit IgG were
15 also prepared. These conditioned media were subjected to Western blotting to
16 determine the types of Aβ-species present in the media, and used to treat
17 differentiated A1 cells for 16 h.

18 **Determination of reactive oxygen species (ROS) level in a neuronal cell**
19 **line culture**

20 ROS level in A1 neuronal cell line was evaluated by Dihydroethidium
21 (DHE) staining, as described previously (Castro et al., 2009) with some
22 modifications. Briefly, A1 cells were cultured on a glass chamber slide and
23 differentiated. DHE (Millipore) was dissolve in DMEM to make a final
24 concentration of 10 μM. After receiving appropriate treatment the cells were

washed with PBS, DHE containing DMEM was added to the cell culture, and incubated in a humid condition at 37°C temperature and 5% CO_2 for 5 min. Then the cells were washed with PBS on an orbital shaker, at room temperature for 5 min. The images were captured immediately using a confocal microscope at excitation and emission wavelengths of 518 and 606 nm, respectively. The whole procedure of DHE staining was done in a dark condition.

Statistical analysis

The results are expressed as mean ± SEM of at least three independent experiments. Statistical analysis for comparing mean values was performed using one-way ANOVA, followed by Scheffe's post hoc test, or *student's t* test. p values < 0.05 indicate statistical significance.

Result

Effects of LPC on Aβ-induced toxicity on a neuronal cell line. In order to investigate the effects of LPC on Aβ-induced toxicity, a neuronal cell line (A1) was treated with LPC (10 μM) in the presence or absence of $Aβ_{1-42}$ (10 μM) for 24 h. The morphological analysis showed that in medium-only and LPC-only treated conditions, A1 had a large cell body with processes (Figure 1A). Few cells with small and round shaped body were found in those conditions. In $Aβ_{1-42}$-only condition, small and round shaped cell number was increased with a reduction of cell density. In $Aβ_{1-42}$ with LPC-treated condition, small and round shaped cell number was increased, and the cell density decreased further compared to $Aβ_{1-42}$-only condition (Figure 1A).

To investigate further, the effects of LPC and $Aβ_{1-42}$ on A1 cell viability were analyzed. The results of MTT cell viability assay demonstrated that LPC did not decrease A1 cell viability up to 10 μM concentration (Figure 1B). At higher concentrations, such as above the concentration of 20 μM, LPC significantly decreased cell viability (data not shown). The dose-dependent experiment further revealed that $Aβ_{1-42}$ significantly decreased A1 cell viability starting from 5 μM (Figure 1C). Addition of LPC (10 μM) augmented such effect, and significant reduction of the viability was observed from 2 μM concentration of $Aβ_{1-42}$ (Figure 1C). Moreover, the viability was significantly decreased in $Aβ_{1-42}$ with LPC (10 μM) condition compared to $Aβ_{1-42}$-only of same concentration, starting from 5 μM concentration of the peptide (Figure 1C). This augmenting effect of LPC on $Aβ_{1-42}$-induced decrease cell viability was dose dependent, which was evident from 5 μM concentration of LPC (Figure 1D).

1 Next, we investigated the effects of LPC and Aβ_{1-42} on LDH release in
2 A1 cell culture. Dose dependent experiments showed that after 24 h treatment,
3 neither LPC nor Aβ_{1-42} up to 10 μM concentration, had any effects on LDH
4 release in the culture media (Figure 1E). Even, a combined treatment of Aβ_{1-42}
5 (5 μM) and LPC (up to 10 μM) failed to affect LDH release from the cells (Figure
6 1F)
7 .**Effects of LPC on Aβ_{1-42}-induced apoptosis in a neuronal culture**. Next, we
8 investigated whether the reduction of A1 cell viability was due to apoptotic cell
9 death. A1 cells were treated with medium-only, LPC-only (10 μM), or Aβ (5 μM)
10 in the presence or absence of LPC for 24 h. Evaluation of apoptotic cell death
11 by TUNEL assay revealed that about 5% cells were TUNEL positive in medium-
12 only or LPC-only condition, which was significantly increased to 13.3±2.6 % in
13 Aβ_{1-42}-only treated condition (Figure 2A and 2B). Interestingly, compared to Aβ_{1-}
14 $_{42}$-only, the percentage of TUNEL positive cells was significantly increased in
15 Aβ_{1-42} with LPC-treated condition to 40.7±6.6 % (Figure 2A and 2B).
16 **Effects of LPC on Aβ_{1-42} induced expression of proteins implicated in**
17 **apoptosis.** To explore the underlying mechanism, the expression of proteins
18 implicated in apoptosis pathways including Bax and Bcl-2 were investigated. A1
19 cells were treated with medium-only, LPC-only (10 μM), or Aβ (5 μM) in the
20 presence or absence of LPC for 8 h and 24 h. Western blotting result showed
21 that Bcl-2 protein level was not changed by any treatment at 8h (Figure 3A and
22 3D). It was similarly decreased by Aβ_{1-42}-only and Aβ_{1-42} with LPC-treated
23 condition at 24 h (Figure 3B and 3D). On the other hand, Bax protein level was
24 started to increase in Aβ_{1-42}-only or Aβ_{1-42} with LPC-treated condition from 8 h

(Figure 3A and 3C), however, that effect was not statistically significant. After 24 h treatment, Bax protein level was increased in both LPC- and $A\beta_{1-42}$-only-treated condition. Compared to $A\beta_{1-42}$-only condition, Bax protein level was significantly in $A\beta_{1-42}$ with LPC-treated condition (Figure 3B and 3C). As a result, the ratio of Bax to Bcl-2 was significantly increased in $A\beta_{1-42}$ with LPC-treated condition compared to $A\beta_{1-42}$-only condition (Figure 3E).

Effects of LPC on $A\beta_{1-42}$-induced intrinsic pathway of apoptosis. The ratio of Bax to Bcl-2 proteins plays an important role in the activation of intrinsic pathway of apoptosis (Almeida et al., 2000; Kluck et al., 1997). As Bax to Bcl-2 ratio was increased in $A\beta_{1-42}$ with LPC-treated condition, we investigated further about the apoptosis pathway that was activated in this condition. A1 cells were treated with medium-only, LPC-only (10 μM), or Aβ (5 μM) in the presence or absence of LPC for 24 h. Western blotting result revealed that, although $A\beta_{1-42}$-only or $A\beta_{1-42}$ with LPC-treated condition increased the protein levels of pro-caspase8, the active form of caspase8 was not detectable in any conditions (Figure 4A and 4B). In the case of caspase9, LPC-only, $A\beta_{1-42}$-only or $A\beta_{1-42}$ with LPC-treated condition similarly increased pro-form of the enzyme (Figure 4A and 4C). However compared to $A\beta_{1-42}$-only, active form of caspase9 was significantly increased in $A\beta_{1-42}$ with LPC-treated condition (Figure 4A and 4D).

Role of $A\beta_{1-42}$ oligomers on LPC-induced increased toxicity in a neuronal cell line culture. To analyze further about the underlying mechanisms, we examined about the effect of $A\beta_{1-42}$ oligomer formation on the neurotoxicity. In our previous report, we have demonstrated that LPC increased $A\beta_{1-42}$ oligomer formation in a fibril formation buffer (Sheikh and Nagai, 2011). As the oligomer is

1 considered as most toxic species of $A\beta_{1-42}$ (Malaplate-Armand et al., 2006), we
2 investigated whether LPC can increase it in the culture condition. $A\beta_{1-42}$ (10 μM)
3 was added to DMEM medium alone, DMEM medium containing LPC (10 μM)
4 and incubated at 37°C. Oligomer formation in the culture condition was
5 evaluated by dot blot immunoassay using an oligomer specific antibody. The
6 time course result showed that $A\beta_{1-42}$ aggregated to form oligomers in cell
7 culture medium after 24 h incubation (Figure 5A and 5B). LPC augmented that
8 $A\beta_{1-42}$ oligomer formation process, which appeared after 4 to 8 h of incubation
9 (Figure 5A and 5B). Oligomer formation was further evaluated by Western
10 blotting using Aβ-specific monoclonal antibody. The results showed that after
11 adding $A\beta_{1-42}$ in DMEM medium, the molecular size of the major peptide band
12 was about 5 KDa, suggestive of peptide monomer. A few proportion of the
13 peptide was found to exist as dimer, trimer and tetramer (Figure 5C, lane 3 and
14 4). After 8 h incubation, a smear-like signal from 35 KDa to more than 180 KDa,
15 suggestive of Aβ-oligomers, appeared in $A\beta_{1-42}$ with LPC condition,
16 concomitantly low-molecular-weight $A\beta_{1-42}$ signal (less than 10 KDa size) was
17 decreased (Figure 5C, lane 6). In Aβ-only condition, oligomers were detectable
18 from 24 h incubation, however, the level was low compared to corresponding
19 $A\beta_{1-42}$ with LPC condition (compare lane 7 and 8 of Figure 5C).
20 Then the neurotoxic property of increased $A\beta_{1-42}$ oligomers formation by
21 LPC was investigated. $A\beta_{1-42}$ (10 μM) was allowed to form oligomers in the cell
22 culture media (DMEM) for 8 h in the presence or absence of LPC (10 μM). Then
23 the oligomers were removed from the culture media by immunoprecipitation
24 using oligomer specific antibody (Figure 5D). Such conditioned media were

used to treat A1 cells for 16 h, and the activation of caspase9 was investigated by Western blotting. The results showed that without immunoprecipitation, active form of caspase9 level was increased in A$\beta_{1\text{-}42}$ with LPC compared to A$\beta_{1\text{-}42}$-only-treated condition (Figure 5E and 5F). Interestingly, removing early formed oligomers (after 8 h incubation) from A$\beta_{1\text{-}42}$ with LPC media by immunoprecipitation decreased active form of caspase9 level compared to without immunoprecipitation (Figure 5E and 5F).

Next, the cell viability was analyzed by MTT assay. The result demonstrated that in the case of non-immunoprecipitation or immunopercipitation by normal goat IgG conditions, A$\beta_{1\text{-}42}$-only decreased the cell viability, which was further decreased significantly in A$\beta_{1\text{-}42}$ with LPC-treated condition (Figure 5G). Removal of oligomers by immunoprecipitation abolished such difference between A$\beta_{1\text{-}42}$-only and A$\beta_{1\text{-}42}$ with LPC-treated condition (Figure 5G).

Effects of LPC on A$\beta_{1\text{-}42}$-induced reactive oxygen species production in a neuronal cell line culture.

Previous studies showed that Aβ oligomers can increase reactive oxygen species (ROS) level in neuronal cells and endothelial cells (He et al., 2013; Wan et al., 2014). Moreover, ROS has been shown to activate intrinsic pathway of apoptosis (Holtz et al., 2006). As LPC increased oligomer formation, and activated caspase9, we investigated whether LPC increased A$\beta_{1\text{-}42}$-induced ROS production. The results of DHE assay showed that A$\beta_{1\text{-}42}$ increased ROS level in A1 cells (e of Figure 6A, and 6B). Interestingly, compared to A$\beta_{1\text{-}42}$-only treatment, A$\beta_{1\text{-}42}$ with LPC treatment further increased ROS level in A1 cells (e

and g of Figure 6A, and Figure 6B).

Discussion

In the previous study, we have demonstrated that LPC increases the rate of $A\beta_{1-42}$ oligomer formation in an in vitro fibril formation system (Sheikh and Nagai, 2011). In the current study, similar effect of LPC was observed in a neuronal cell culture condition. Such increased rate of $A\beta_{1-42}$ oligomer formation appeared to potentiate the neurotoxic effect of the peptide. Since Aβ oligomer-induced neurodegeneration is considered to be the main cause that initiates AD-like pathological changes in the brains (Lorenzo and Yankner, 1994; Selkoe, 1991), LPC might have an important role in such condition.

Neuronal loss in the affected area is the hall mark of AD pathology, and a clear understanding of the underlying mechanism could be valuable to devise an effective disease modifying strategy. In the AD brains, the deposition of Aβ peptide is always found in the area of neurodegeneration, suggesting the significance of the peptide in the process (Lorenzo and Yankner, 1994; Selkoe, 1991; Selkoe, 1996). In vitro cell culture studies confirmed the neurodegenerative effects of Aβ (Yankner et al., 1990), and apoptosis is suggested to be a possible mechanism of neurodegeneration (Loo et al., 1993; Yang et al., 2009). However, detail mechanisms regarding the activation of the pathways in Aβ-induced apoptosis are not fully understood (Dickson, 2004). In this study, we have found that $A\beta_{1-42}$ specifically affected the protein levels of Bcl-2 and Bax resulting increased Bax/Bcl-2 ratio, increased both pro and active caspase9, and consequently increased A1 neuronal cell apoptosis without affecting LDH release from the cells. A change of Bax/Bcl-2 ratio and

subsequently activation of caspase9 play a central role in apoptosome-dependent intrinsic pathway of apoptosis (Franklin, 2011; Lindsay et al., 2011; Würstle et al., 2012). Although we did not investigated the early events, such as cytochrome C release, a change of Bax/Bcl-2 ratio and activation of caspase9 are suggesting that intrinsic pathway of apoptosis might be activated in this condition. Moreover, in the brains of AD patients, Bax expression is increased in Aβ-associated dystrophic neurons (Tortosa et al., 1998), supporting the involvement of intrinsic apoptosis pathway-related mechanism in the process. Interestingly, without affecting LDH release, LPC significantly increased Aβ-induced Bax and active caspase9 levels, and consequently increased apoptotic cell number. These results are suggesting that the modulation of the proapoptotic proteins of intrinsic apoptosis pathway could be the basis of the enhancing effects of LPC on Aβ-induced neuronal apoptosis.

Reports have been demonstrated that LPC can induce apoptotic cell death in smooth muscle cell (SMC) and endothelial cell (EC) culture (Hsieh et al., 2000; Takahashi et al., 2002). Hence, there is a possibility that LPC could directly induced apoptosis of A1 cells, and increased apoptosis in $A\beta_{1-42}$ with LPC-treated condition might just the combined result of two independent effects. In the case of SMC and EC, high concentration of LPC is required to induce apoptosis in a serum free condition (Hsieh et al., 2000; Takahashi et al., 2002). Here also, only high concentration of LPC (20 μM and above) decreased the viability of A1 cells. So the enhancing effect of LPC at low concentration (10 μM and below) on $A\beta_{1-42}$-induced apoptosis might not be an $A\beta_{1-42}$-independent effect, rather a modulating effect on $A\beta_{1-42}$-induced apoptosis mechanism.

1 Although controversial, many of the effects of LPC on the cellular events
2 including apoptosis are shown to be mediated through G2A, a G-protein
3 coupled receptor (Hsu et al., 2011; Kabarowski et al., 2002; Lin and Ye, 2003;
4 Obinata and Izumi, 2009; Sheikh et al., 2009). However, in A1 cells, we have
5 found that the expression of G2A is very low, and any of the stimulation did not
6 increased that expression, as determined by Western blotting (data not
7 shown).Therefore the apoptosis enhancing effects at low concentration might
8 be mediated through augmentation of Aβ-induced apoptosis process. However,
9 as LPC alone increased the level of Bax, a direct effect on apoptosis could not
10 be ignored (Hsu et al., 2011).
11 Reports have been shown that while aggregated state of Aβ peptide
12 induces the death of neuronal cells, soluble non-aggregated Aβ peptide does
13 not have such ability, rather it increases the neurite growth (Cotman et al.,
14 1994). Such findings are indicating that the aggregation processes have a great
15 role to determine the neurodegenerative potentials of Aβ. In the previous report,
16 we have demonstrated that LPC induces $A\beta_{1-42}$ aggregation at a lower
17 concentration, and at a faster rate (Sheikh and Nagai, 2011). Consistently,
18 similar aggregation enhancing effects were found in cell culture condition, and
19 the aggregated oligomers possibly interacted with A1 cells at an earlier time
20 point. Such interaction might be important, as removing early formed oligomers
21 from the culture medium decreased caspase9 activation and eliminated
22 cytotoxicity enhancing effect. Moreover, compared to Aβ-only condition,
23 removing oligomers by immunoprecipitation decreased Aβ peptide
24 concentration in $A\beta_{1-42}$ with LPC-treated condition. Still the toxic effect was

1 similar like Aβ_{1-42} only condition, showing the ability of LPC to increase Aβ_{1-42}-
2 induced toxicity at lower concentration.
3 LPC is produced in the cell from phosphatidylcholine by PLA2 activity
4 (Steinbrecher et al., 1984). In AD condition, PLA2 activity is shown to be altered
5 (Schaeffer et al., 2009; Stephenson et al., 1996). Postmortem analysis revealed
6 that at an early stage, PLA2 activity is decreased in the brains of AD patient
7 (Schaeffer et al., 2009). As the disease advances, activity are demonstrated to
8 be increased (Schaeffer et al., 2009; Stephenson et al., 1996). Such increased
9 PLA2 activity is suggested to induce an inflammatory condition by activating
10 arachidonic acid pathway. However, as a product of PLA2 activity, LPC level
11 might be changed in such condition (Wender et al.; 1998), which could directly
12 the neuroinflammation and neurodegeneration (Sheikh et al., 2009). Therefore,
13 in addition to activation of arachidonic acid pathway, increased PLA2 activity in
14 AD condition might also increase LPC production, and subsequently Aβ
15 oligomer formation, neuroinflammation and neurodegeneration.
16 Aβ oligomers have been shown to increase ROS level in endothelial
17 cells and neuronal cells (He et al., 2013; Wan et al., 2014). It also can regulate
18 Bax and Bcl-2 protein expression (Marques et al., 2003), and activation of
19 intrinsic pathway of apoptosis (Picone et al., 2009). Furthermore, increased
20 ROS level has the ability to induce apoptosis by activating intrinsic pathway
21 (Holtz et al., 2006). Here we have demonstrated that LPC increased Aβ_{1-42}-
22 induced ROS level, along with increased oligomer formation, apoptosis and
23 activation of caspase9. Such results are suggesting that oligomer mediated
24 increased ROS level might be an initial event that leads to activation of intrinsic

pathway of apoptosis through regulation of Bax and Bcl-2 protein levels in this condition.

Conclusion

Thus our results showed that increased LPC level induced Aβ peptide oligomer formation and subsequently neurodegeneration. Because Aβ-induced neurodegeneration is the main pathological feature of AD, LPC might play a role in the pathological process of the disease.

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Figure Legends:

Figure 1. Effects of LPC on $A\beta_{1-42}$-induced toxicity on a neuronal cell line. A1 neuronal cell line was treated with medium-only, LPC-only (10 μM), or $A\beta_{1-42}$ (10 μM) in the presence or absence of LPC (10 μM) for 24 h. The changes in cell morphology were analyzed by cell culture microscopy, and photomicrographs of the culture are show in (A). A1 neuronal cells were treated with indicated concentrations of LPC-only, $A\beta_{1-42}$-only, $A\beta_{1-42}$ in the presence of LPC (10 μM), or LPC in the presence of $A\beta_{1-42}$ (5 μM) for 24 h, and cell viability was evaluated by MTT assay. The dose-dependent effects of LPC-only are shown in (B). The dose-dependent effects of $A\beta_{1-42}$-only, and $A\beta_{1-42}$ in the presence of LPC (10 μM) are shown in (C), and the dose dependent effects of LPC in the presence of $A\beta_{1-42}$ (5 μM) are shown in (D). The data was expressed as % control, where cells of a normal culture was served as such. A1 cells were treated with indicated concentrations of $A\beta_{1-42}$-only, LPC-only, or LPC in the presence of $A\beta_{1-42}$ (5 μM) for 24 h, and LDH release in the culture medium was evaluated. The dose dependent effects of $A\beta_{1-42}$-only or LPC-only on LDH release are shown in (E), and the dose dependent effects of LPC in the presence of $A\beta_{1-42}$ (5 μM) are shown in (F). The data was expressed as % control, where medium-only treated culture was served as such. The average ± SEM of at least 3 independent experiments are shown here. Statistical analysis was performed by one-way ANOVA, followed by Scheffe's post hoc test, and the significance is denoted as follows, $^{*}p<0.05$ vs medium-only treated condition, $^{\#}p<0.05$ vs corresponding $A\beta_{1-42}$-only, and $^{\dagger}p<0.05$ vs $A\beta_{1-42}$ 5 μM condition.

1 **Figure 2. Effects of LPC on Aβ_{1-42}-induced apoptosis in a neuronal cell**
2 **line.** A1 neuronal cell line was treated with medium-only, LPC-only (10 μM), or
3 Aβ (5 μM) in the presence or absence of LPC for 24 h. Apoptotic cells in the
4 culture were detected by TUNEL assay, as described in the Materials and
5 Methods. Hoechst nuclear staining was done to count the total cells in a field.
6 Representative photomicrographs of Hoechst staining and TUNEL positive cells
7 in the cultures are shown in (A). TUNEL positive cells were counted in 5
8 randomly selected high power fields, averaged and expressed as % of total
9 cells in the field. The average ± SEM data of 3 independent experiments are
10 shown in (B). Statistical analysis was performed by one-way ANOVA, followed
11 by Scheffe's post hoc test, and the significance is denoted as follows, $^{*}p<0.05$ vs
12 medium alone, $^{\dagger}p<0.01$ vs medium alone, $^{\#}p<0.01$ vs Aβ_{1-42} condition.
13 **Figure 3. Effects of LPC and Aβ_{1-42} on apoptosis related protein level in a**
14 **neuronal cell line.** A1 neuronal cell line was treated with medium-only, LPC-
15 only (10 μM), or Aβ_{1-42} (5 μM) in the absence or presence of LPC for 8 h and 24
16 h. Bax and Bcl-2 protein levels in the cultured cells were evaluated by Western
17 blotting, as described in the Materials and Methods. A representative Western
18 blotting data of 8 h (A) and 24 h (B) treatments are shown. β-Actin served as
19 loading control. β-Actin normalized densitometry data of Bax (C) and Bcl-2 (D),
20 and their ratio (E) of 8 h and 24 h treatment are shown. Densitometry data
21 presented here as mean ± SEM of at least 3 independent experiments, and
22 shown as % control, where medium-only condition was served as such.
23 Statistical analysis was performed by one-way ANOVA, followed by Scheffe's
24 post hoc test, and the significance is denoted as follows, $^{*}p<0.05$ vs Aβ_{1-42}-only

condition, and $^{\#}p<0.05$ vs medium only condition.

Figure 4. Effects of LPC and $A\beta_{1-42}$ on apoptosis related enzymes activation in a neuronal cell line. A1 neuronal cell line was treated with medium-only, LPC-only (10 μM), or $A\beta_{1-42}$ (5 μM) in the absence or presence of LPC for 24 h. Caspase8 and caspase9 proteins in the cultured cell lysates were analyzed by Western blotting, as described in the Materials and Methods. A representative Western blotting data is shown in (A). β-Actin served as loading control. β-Actin normalized densitometry data of pro-caspase8, pro-caspase9 and active caspase9 are shown in (B), (C) and (D), respectively.

Figure 5. Role of LPC-induced $A\beta_{1-42}$ oligomer formation on neurotoxicity. $A\beta_{1-42}$ (10 μM) was incubated in DMEM medium containing 1% FBS in the absence or presence of LPC (10 μM) for indicated time at 37°C. The oligomers formed in the media were evaluated by dot blot immunoassay and Western blotting, as described in the Materials and Methods. A representative dot blot immunoassay and the average densitometric analysis of 3 independent experiments are shown in (A) and (B), respectively. Western blotting data are shown in (C). In (C), lane 1 is for protein size marker, lane 2 is for 1% FBS/DMEM, lane 3, 5 and 7 are for $A\beta_{1-42}$-only (10 μM), and lane 4, 6 and 8 are for $A\beta_{1-42}$ with LPC (10 μM); where samples of lane 3 and 4 were incubated for 0 h, lane 5 and 6 for 8 h, and lane 7 and 8 for 24 h. (D) In DMEM, LPC, $A\beta_{1-42}$ only, or $A\beta_{1-42}$ with LPC was added to make final concentration of each molecule to 10 μM, and incubated at 37°C for 8 h. Then the oligomers were removed from the media by immunoprecipitation using an oligomer-specific antibody (A11). To evaluate oligomeric and other species of $A\beta_{1-42}$, Western

1 blotting was done using Aβ specific antibody. The samples without
2 immunoprecipitation, or immunoprecipitated with normal rabbit IgG were served
3 as control. Differentiated A1 cells were treated with the conditioned media,
4 prepared as described above, for 16 h, and levels of active form of caspase9
5 were evaluated by Western blotting. A representative Western blotting and its
6 densitometric analysis are shown in (E) and (F), respectively. (G) After
7 treatment with the conditioned media for 16 h, A1 cell viability was further
8 evaluated by MTT assay. Non-immunoprecipitation and normal rabbit IgG-
9 immunoprecipitation conditions were used as controls. The average ± SEM data
10 of 3 independent experiments are presented here, calculated as % control,
11 where cells of a normal culture served as such. Statistical analysis was done by
12 one-way ANOVA, followed by Scheffe's post hoc test, and the significance is
13 denoted as follows, $^{*}p<0.05$ vs corresponding $A\beta_{1-42}$-only condition.
14 **Figure 6. Effects of LPC on $A\beta_{1-42}$-induced generation of reactive oxygen**
15 **species (ROS) in a neuronal cell line.** In DMEM, LPC, $A\beta_{1-42}$, or $A\beta_{1-42}$ with
16 LPC was added to make final concentration of each molecule to 10 μM, and
17 incubated at 37°C for 8 h. Then the conditioned media were used to treat A1
18 cells for 30 min, and the levels of ROS was evaluated by Dihydroethidium
19 staining and acquiring the photomicrographs using a confocal microscope.
20 Representative photomicrographs of medium-only (a and b), LPC (c and d),
21 $A\beta_{1-42}$ (e and f) and $A\beta_{1-42}$ with LPC (g and h) are shown in (A), where the
22 localization of DHE stained nuclei (a, c, e and g) were determined by bright-field
23 images (b, d, f and h). Quantified data of average fluorescence intensities of 3
24 independent experiments are shown in (B). Statistical analysis was done by

1 one-way ANOVA, followed by Scheffe's post hoc test, and the significance is

2 denoted as follows, $^{*}p<0.05$ vs medium-only condition, $^{\#}p<0.05$ vs $A\beta_{1-42}$-only

3 condition.

Figure 1

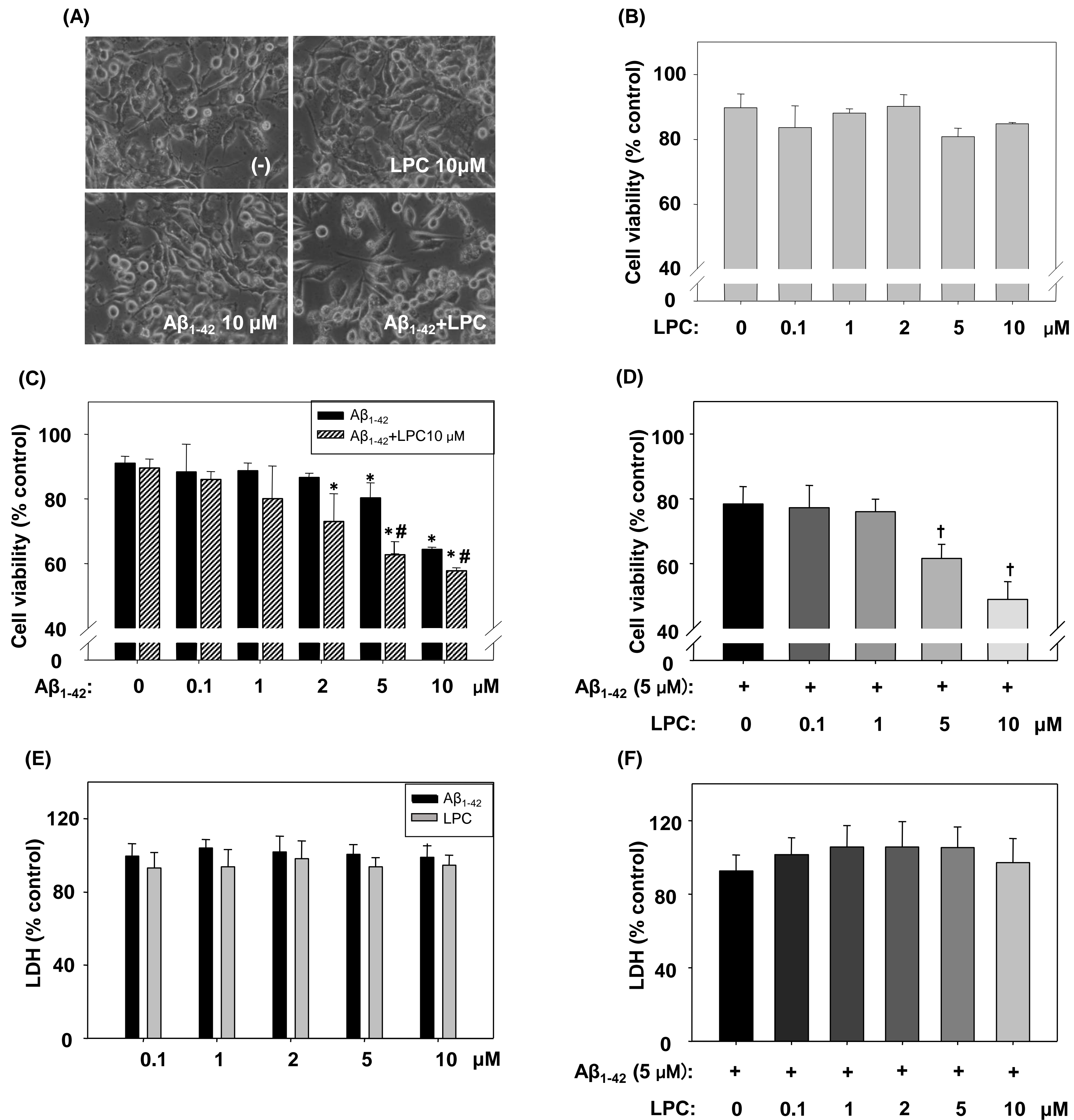
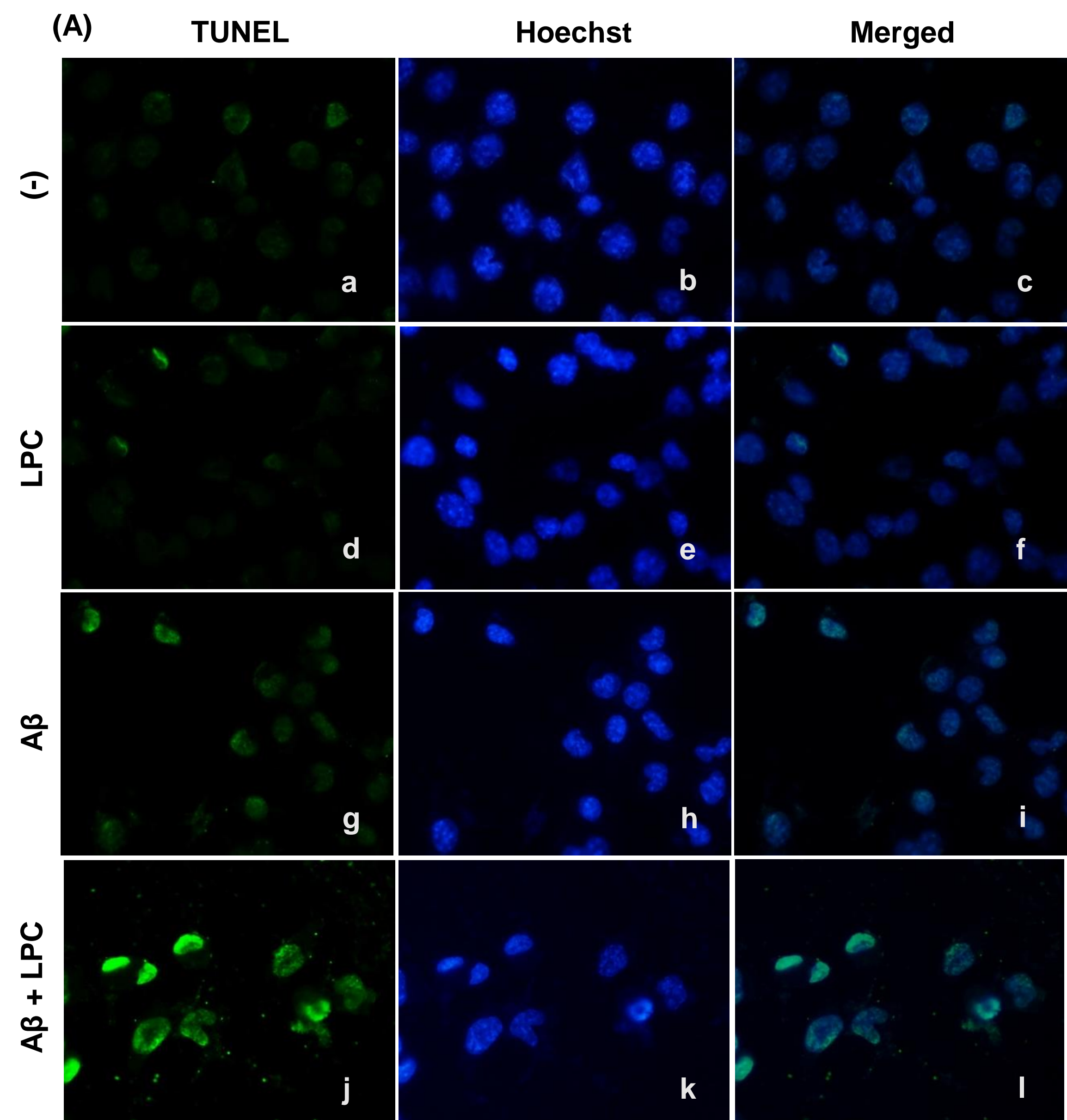


Figure 2



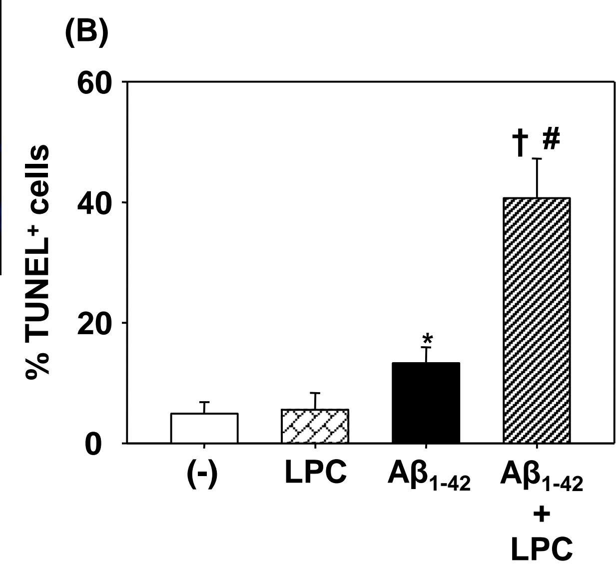
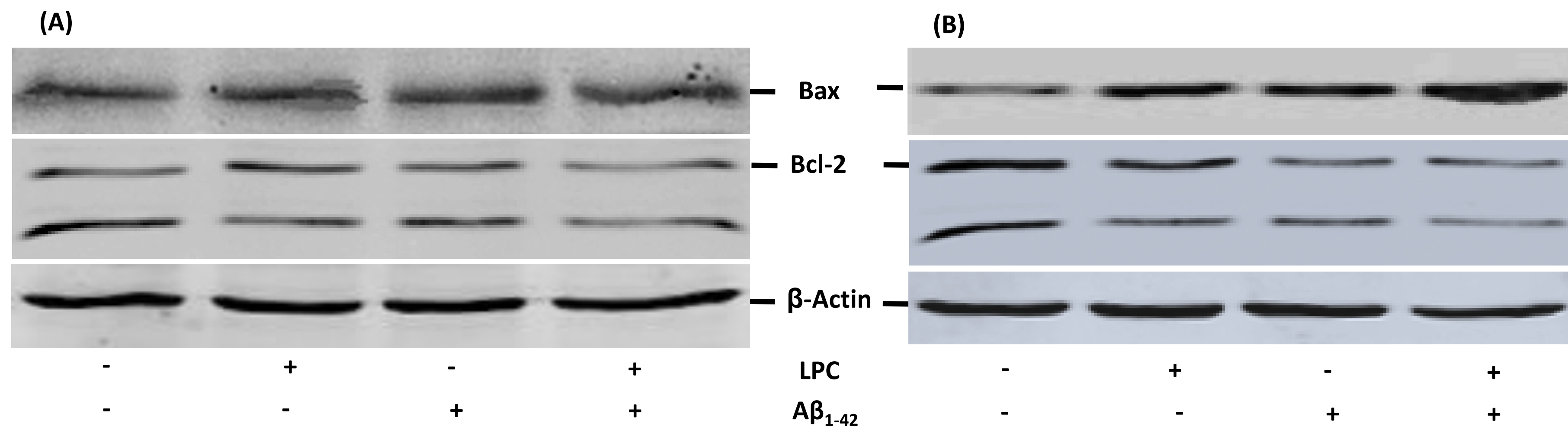
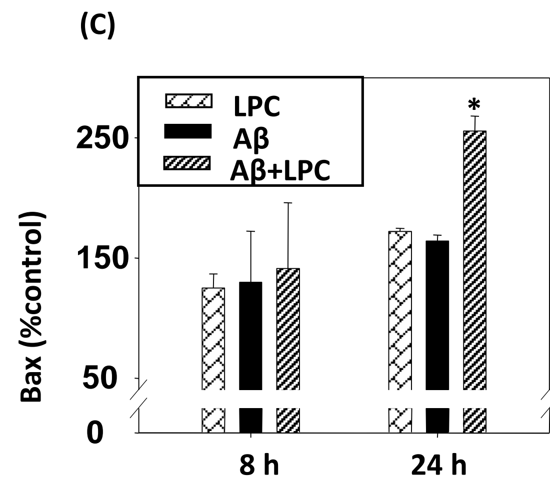
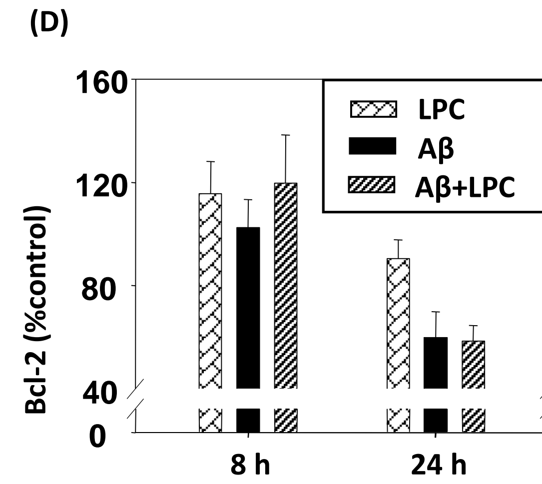


Figure 3







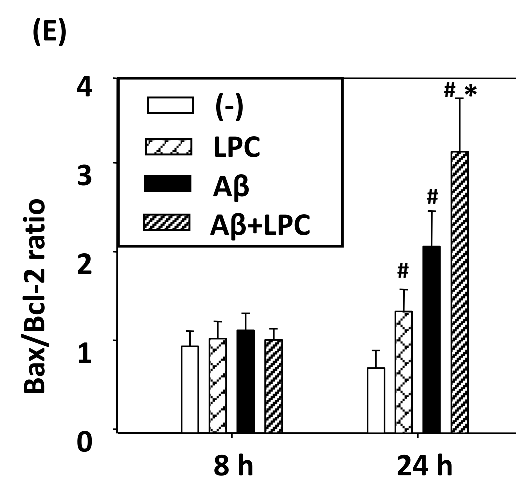
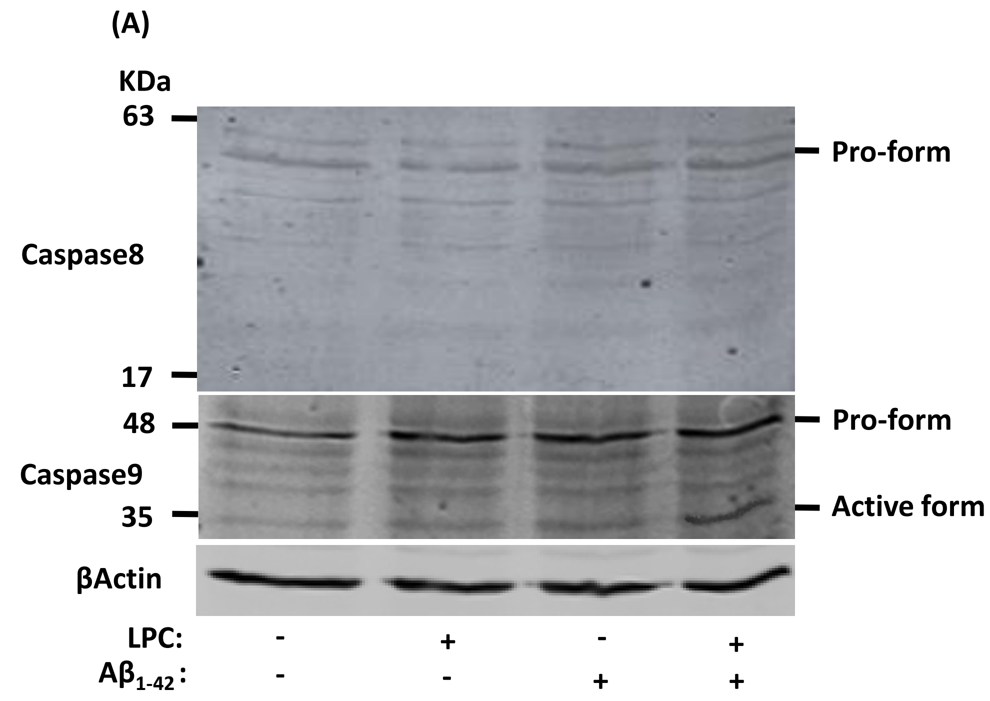
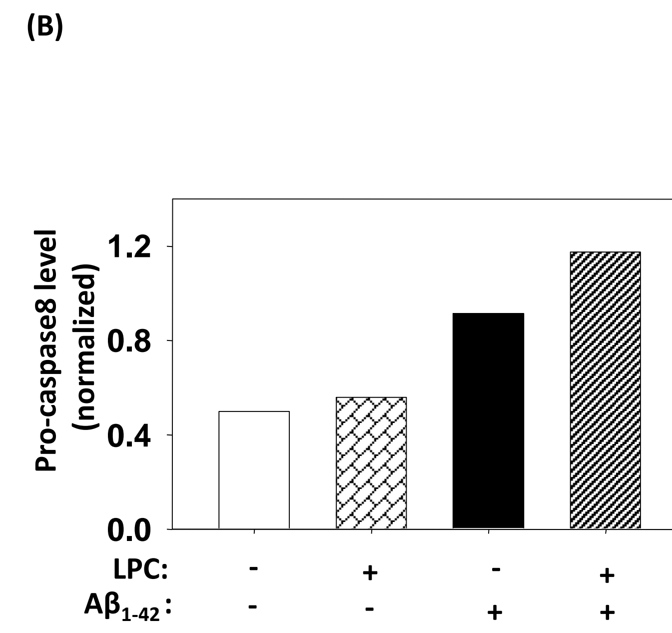
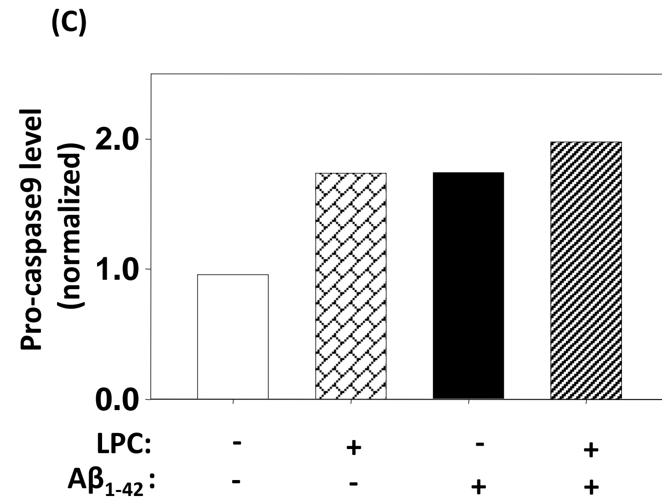


Figure 4







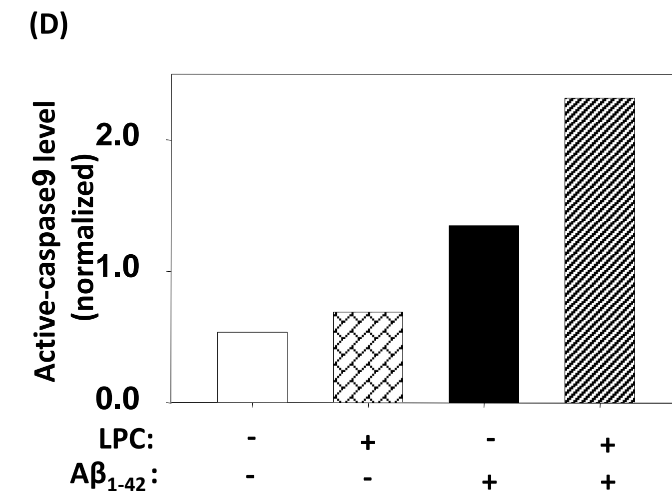
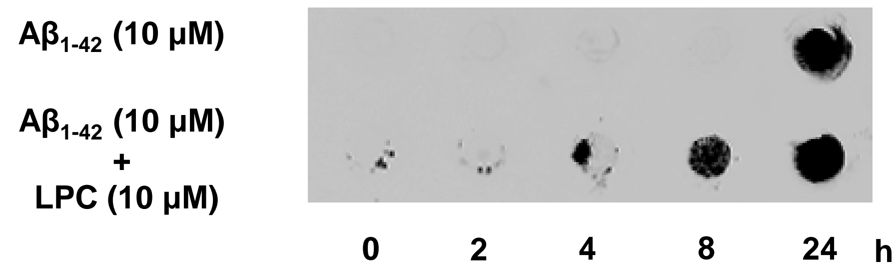


Figure 5

(A)



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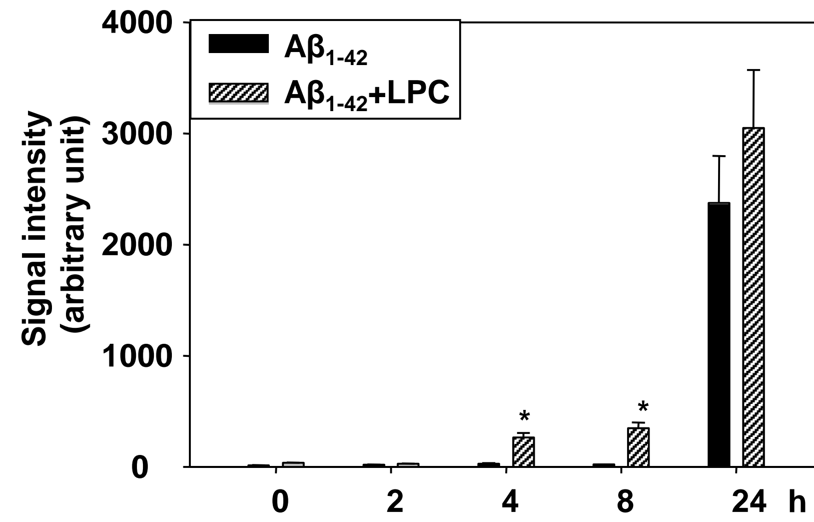
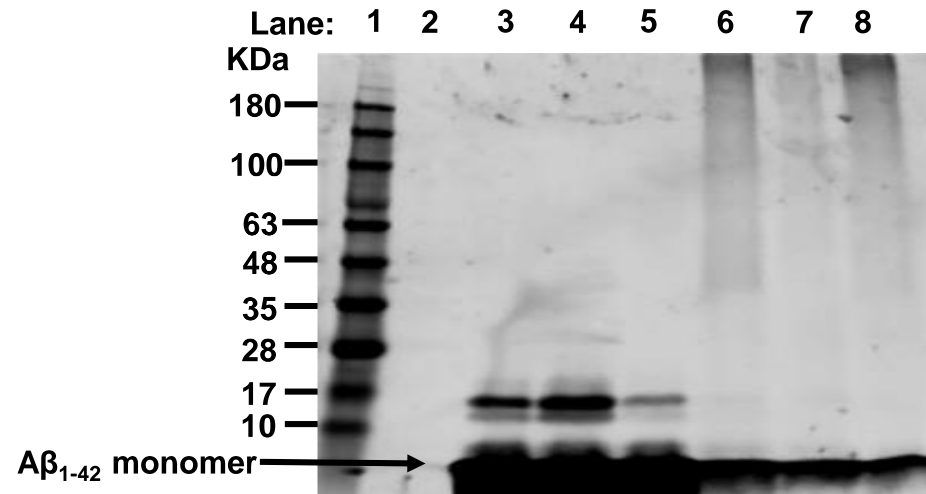


(C)



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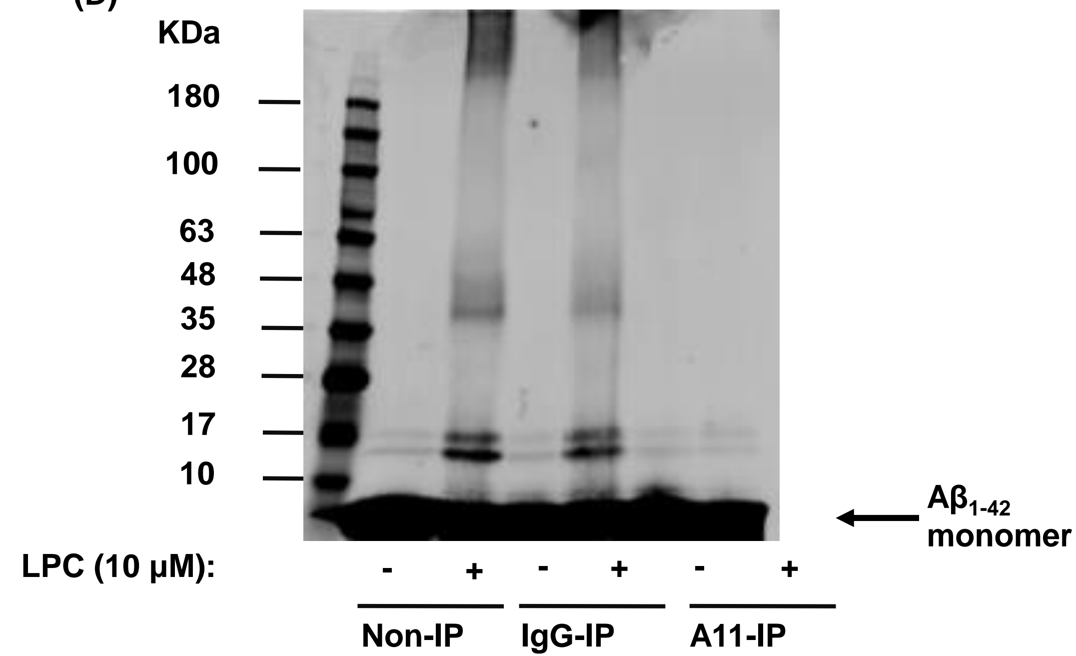

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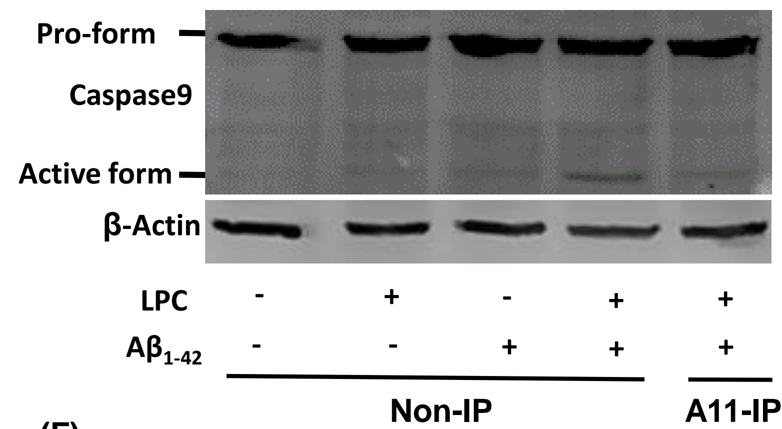

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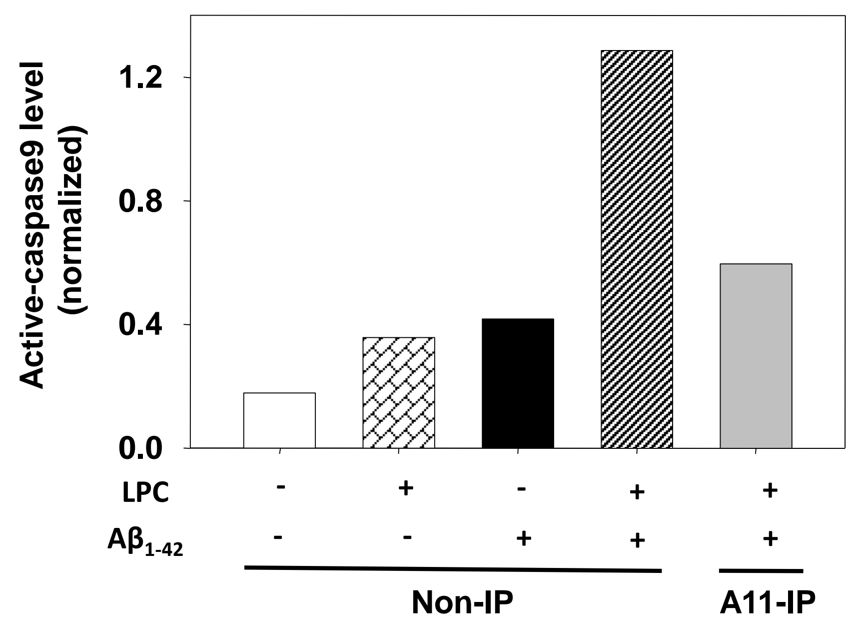


(G)

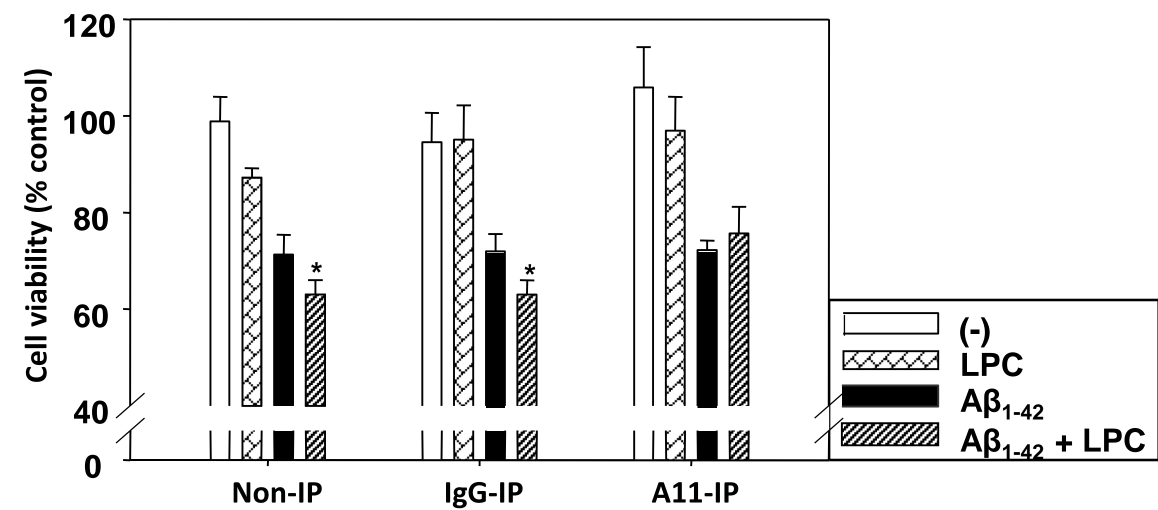
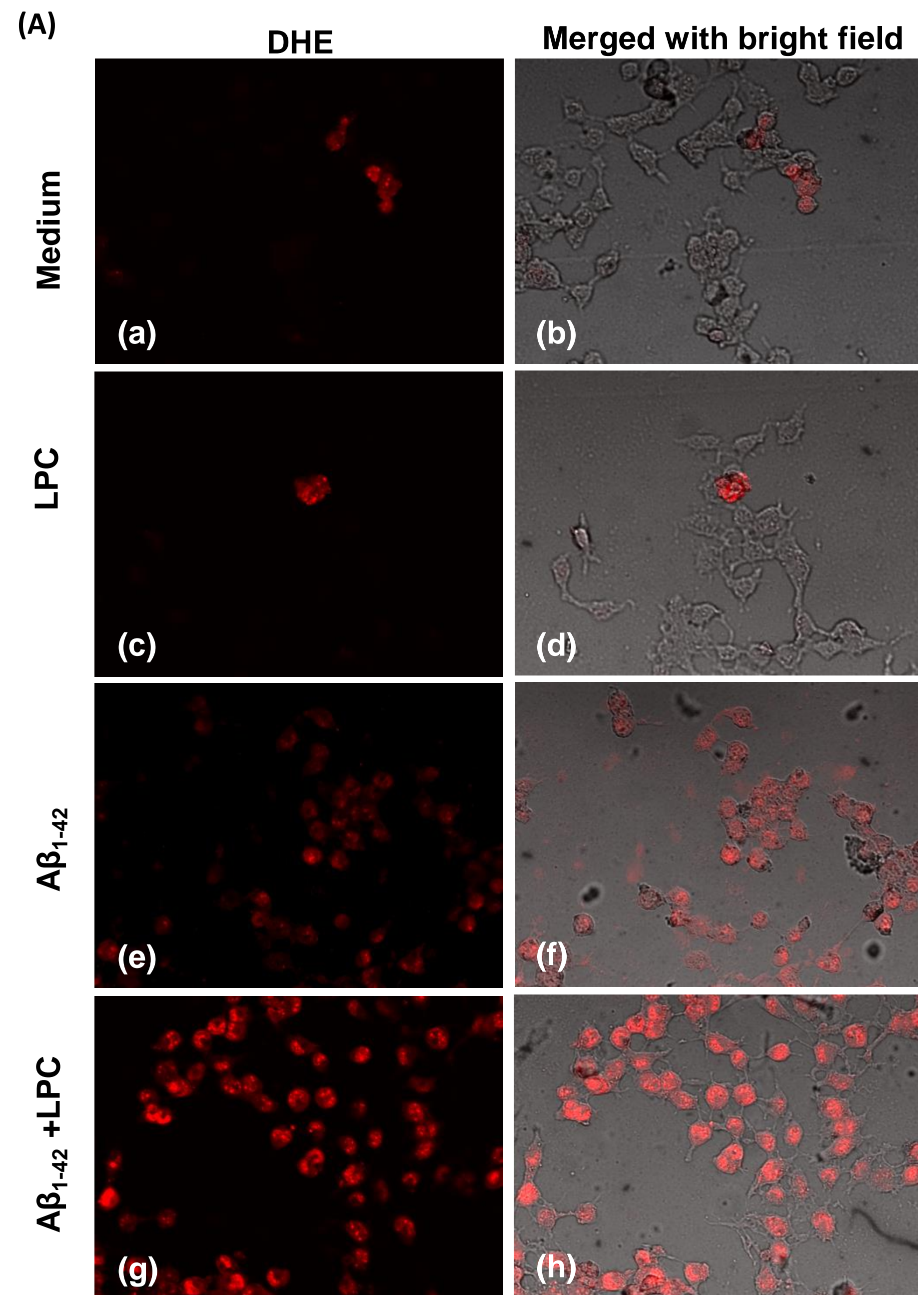


Figure 6

(A)



(B)