Original Article

Effects of lysophosphatidylcholine on β-amyloid-induced neuronal apoptosis

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Aim: We have investigated the effects of lysophosphatidylcholine (LPC), a product of lipid peroxidation, on $A\beta_{1-42}$-induced SH-SY5Y cell apoptosis.
Methods: The viability of cultured SH-SY5Y cells was measured using a CCK-8 kit. Apoptosis was determined by Chip-based flow cytometric assay. The mRNA transcription of Bcl-2, Bax, and caspase-3 were detected by using reverse transcription and real-time quantitative PCR and the protein levels of Bax and caspase-3 were analyzed by Western blotting. The cytosolic calcium concentration of SH-SY5Y cells was tested by calcium influx assay. G2A expression in SH-SY5Y cells was silenced by small interfering RNA.
Results: Long-term exposure of SH-SY5Y cells to LPC augmented the neurotoxicity of $A\beta_{1-42}$. Furthermore, after LPC treatment, the Bax/Bcl-x_L ratio and the expression levels, as well as the activity of caspase-3 were, elevated, whereas the expression level of TRAF1 was reduced. Because LPC was reported to be a specific ligand for the orphan G-protein coupled receptor, G2A, we investigated LPC-mediated changes in calcium levels in SH-SY5Y cells. Our results demonstrated that LPC can enhance the $A\beta_{1-42}$-induced elevation of intracellular calcium. Interestingly, $A\beta_{1-42}$ significantly increased the expression of G2A in SH-SY5Y cells, whereas knockdown of G2A using siRNA reduced the effects of LPC on $A\beta_{1-42}$-induced neurotoxicity.
Conclusion: The effects of LPC on $A\beta_{1-42}$-induced apoptosis may occur through the signal pathways of the orphan G-protein coupled receptor.

Keywords: amyloid beta (1–42) peptide; lysophosphatidylcholine; neuronal apoptosis
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Introduction

Alzheimer's disease (AD) is a degenerative disorder of the central nervous system that causes mental deterioration and progressive dementia. Neuropathologic lesions, including senile plaques in the brains of AD patients, accompany AD. The β-amyloid protein (Aβ), a hydrophobic 39 to 43 residue peptide, is the major component of senile plaques. Much accumulated evidence shows that Aβ fibrillar deposition in senile plaques correlates with the progression of cognitive dysfunction in AD patients. Furthermore, Aβ exhibits neurotoxicity both *in vitro* and *in vivo*[1, 2]. Recent studies have demonstrated that the exposure of cultured neurons to Aβ results in neuronal apoptosis[3, 4].

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Abnormal brain phospholipid metabolism is a feature of AD[5, 6]. Products of lipid peroxidation occur early in the progression of AD. Phosphatidylcholine (PC) is the major phospholipid of eukaryotic membranes[7]. The pathological breakdown of phospholipids by oxidation leads to the production of lysophosphatidylcholine (LPC). It has been reported that $A\beta_{1-42}$ causes lipid peroxidation[8]. Current evidence supports the notion that Aβ-induced lipid peroxidation may in part account for neurodegeneration in the AD brain.

The biological effects and signaling properties of LPC have been extensively studied in atherosclerosis-related cells[9]. LPC can induce neuronal sheath demyelination, axonal degeneration and neuronal apoptosis[10, 11], which are common pathological characteristics of neurodegenerative diseases[12, 13]. However, the physiological role of LPC in neuronal cells is not yet fully understood. Because LPC was

recently shown to be the agonist for an orphan G protein-coupled receptor (GPCR)[14], G2A, it will be interesting to determine whether the effects of LPC on $A\beta_{1-42}$-induced neuronal apoptosis occur through signal pathways of the orphan GPCR.

In this study, we evaluated the effects of LPC on the neurotoxicity of $A\beta_{1-42}$ and examined the expression of several apoptosis-related genes in SH-SY5Y cells. In addition, we analyzed second messenger systems, including intracellular concentrations of calcium, in SH-SY5Y cells after LPC treatment. We found that the orphan G-protein receptor G2A may be involved in LPC effects on neuronal apoptosis. We discuss possible molecular mechanisms underlying the effects of LPC on $A\beta_{1-42}$-induced neuronal apoptosis.

Materials and methods

Cell culture and drug treatment Human neuroblastoma SH-SY5Y cells (ATCC, USA) were routinely cultured in DMEM medium with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C. All media and sera were purchased from Invitrogen Inc. $A\beta_{1-42}$ peptide (CPC Scientific Inc, USA) was dissolved in distilled water at a concentration of 1 mmol/L and stored at -20 °C. The peptide stock solution was diluted to the desired concentrations and maintained for 3 days at 37 °C before use. Lysophosphatidylcholine (LPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in DMSO to make a 20 mmol/L stock solution. SH-SY5Y cells were treated with different concentrations of $A\beta_{1-42}$ and LPC, as indicated in the figure legends.

Proliferation analysis of SH-SY5Y cells SH-SY5Y cells were grown overnight in a 96-well plate (5×10^4 cells/mL). Different concentrations of $A\beta_{1-42}$ and LPC in Opti-MEM I medium were added to the cells and incubated for 48 h. Cell viability was measured using a CCK-8 kit according to the manufacturer's instruction (Dojindo Laboratories, Japan). Briefly, 10 μL of CCK-8 solution was added to each well and incubated for 30 min at 37 °C. The absorbance was measured at 450 nm with a GENios microplate reader (Tecan, Austria).

Chip-based flow cytometric assay SH-SY5Y cells were treated with 10 μmol/L $A\beta_{1-42}$ in Opti-MEM I medium for 48 h and then harvested to the density of 1×10^6 cells/mL. An Annexin V FITC Apoptosis Detection Kit (EMD Chemicals Inc, Germany) was used to measure apoptosis according to the manufacturer's protocol. Briefly, 1.25 μL of Annexin V was added in a 500 μL cell suspension and incubated for 10 min at room temperature. The cells were centrifuged and resuspended in 500 μL of 1×binding buffer containing 2 μg/mL Fluorolink-Cy5 streptavidin (Amersham Biosciences, UK) and 1 μmol/L Calcein-AM (Invitrogen Inc., USA). The samples were incubated for another 10 min at room temperature. After centrifugation, the cells were resuspended again in 100 μL of cell buffer (in Cell Fluorescence LabChip Kit) by gentle pipetting. The cell suspension (10 μL) was added into the cell chip and analyzed by the Agilent 2100 Bioanalyzer.

Reverse transcription and real-time quantitative PCR SH-SY5Y cells (1×10^5) were treated with 5 or 10 μmol/L $A\beta_{1-42}$ and 10 μmol/L LPC in Opti-MEM I medium for 48 h in a 6-well plate. Total RNA was extracted with a Trizol reagent (Invitrogen Inc., USA) based on the manufacturer's instructions. RNA was eluted in 20 μL of nuclease-free water and stored at -70 °C before use. cDNA was synthesized using the M-MLV Reverse Transcriptase (Invitrogen Inc, USA) and oligo(dT) primers (Takara Bio Inc, Shiga, Japan). Three μL of mRNA was incubated with 1 μL of oligo(dT) primer, 1 μL of 10 mmol/L dNTPs (Promega, CA, USA) and 7 μL of nuclease-free water for 5 min at 65 °C. The reaction tubes were immediately placed on ice and a mixture solution (4 μL of 5×first-strand buffer, 2 μL DTT, 1 μL of RNAase-out) was added. The reverse transcription was continued by the addition of 1 μL of M-MLV reverse transcriptase and incubation at 37 °C for 50 min. Finally, the reaction was incubated at 70 °C for 15 min. For fluorescence real-time quantitative RT-PCR, β-actin was used as the reference gene. Primers were designed using Primerexpress software and synthesized by Invitrogen Inc (Table 1). Real-time PCR was carried out in 2 μL of PCR buffer, 0.4 μL of 10 μmol/L sense and antisense primers, 0.4 μL of 10 mmol/L deoxyribonucleotides (dNTPs), 0.5 μL of first-strand cDNA, 0.2 μL of SYBR Green and Taq polymerase (Promega, CA, USA); distilled water was added to a total volume of 20 μL. The reaction was incubated at 94 °C for 5 min, followed by 34 cycles at 94 °C for 1 min; 52 °C for 30 s; and 72 °C for 30 s and finally kept at 72 °C for 5 min using the DNA Engine Opticon 2 system (Bio-Rad, USA). At the end of the reaction, a melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. All reactions were performed in triplicate. Relative transcript quantities were calculated and presented with $2^{-\Delta\Delta Ct}$ values.

Western blot analysis SH-SY5Y cells (1×10^5) were treated with 10 μmol/L $A\beta_{1-42}$ and 10 μmol/L LPC in Opti-MEM I medium for 48 h in a 6-well plate. Cells were homogenized in a cell lysis buffer (Beyotime, Jiangsu, China). After the proteins were quantified in the homogenates, an equal

Table 1. The primers used for quantitative PCR to detect the expression of apoptosis-related genes and orphan GPCR G2A.

Gene name	Genebank cDNA accession number	Primer sequence
Bax	NM_004324	Forward: 5′ TTTGCTTCAGGGTTTCATCC 3′ Reverse: 5′ GCCACTCGGAAAAAGACCTC 3′
Bcl-x_L	NM_138578	Forward: 5′ ATGAACTCTTCCGGGATGG 3′ Reverse: 5′ TGGATCCAAGGCTCTAGGTG 3′
Caspase-3	NM_001167	Forward: 5′ TGGAACAAATGGACCTG 3′ Reverse: 5′ ACCACGGCAGGCCTGA 3′
TRAF1	NM_005658	Forward: 5′ GTGTCGGCTGCTCCTTCAA 3′ Reverse: 5′ CAAACACACGCAGCTTCCC 3′
G2A	NM_013345	Forward: 5′ CGCCAAGAAGTGTCCAGAATC 3′ Reverse: 5′ CCTCAATCAGCCTCTTTGCAG 3′
Beta-actin	NM_001101	Forward: 5′ GGACATCCGCAAAGACCTGTA 3′ Reverse: 5′ ACATCTGCTGGAAGGTGGACA 3′

amount of protein (20 μg/well) from each sample was boiled for 5 min in loading buffer (5% mercaptoethanol, 0.05% bromphenol blue, 75 mmol/L Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol). Proteins were separated by 7.5% SDS-PAGE and transferred to PVDF membranes (Millipore, MA,USA) in a glycine/methanol transfer buffer (20 mmol/L Tris base, 0.15 mol/L glycine, and 20% methanol) using the Trans-Blot SD semidry transfer cell system (BioRad, CA, USA). After being blocked with 5% nonfat dried milk in 1×TBS-T buffer (20 mmol/L Tris-HCl, pH 7.5, 137 mmol/L NaCl and 0.05% Tween-20) for 1 h at room temperature, membranes were probed with primary antibodies (rabbit anti-Bax, rabbit anti-cleaved caspase-3, or rabbit anti-β-actin, Cell Signaling Technology, MA, USA) at a 1:1000 dilution in 1×TBS-T buffer, pH 7.6 at 4 °C overnight. The blots were washed and incubated with anti-rabbit secondary antibodies (1:2000) for 1 h at room temperature. Chemiluminescent detection was performed using an ECL Western blotting Detection kit from Amersham Pharmacia (Buckinghamshire, UK).

Caspase-3 activity assay Caspase-3 activity was measured using a caspase-3/CPP32 colorimetric assay kit according to the manufacturer's protocol (Calbiochem, Darmstadt, Germany). Briefly, cells were treated with 10 μmol/L $A\beta_{1-42}$ and 10 μmol/L LPC in Opti-MEM I medium for 48 h in a 6-well plate, washed with PBS and suspended in lysis buffer. The lysates were centrifuged for 5 min at 14000×*g* and the supernatant was stored at -80 °C before use. Protein concentrations were measured by the BCA method. Lysates (20 μg of protein) were incubated at 37 °C for 2 h in 2 mmol/L Ac-DEVD-pNA substrate and caspase-3 activity was measured using an optimal microplate reader (Tecan GENios, Switzerland), in which the substrate cleavage was monitored at 405 nm.

Calcium influx assay The cytosolic calcium concentration of SH-SY5Y cells was measured using a Flexstation imaging plate reader (Molecular Devices Corporation, USA). Cells (1.5×10^5cells/mL) were seeded into 96-well black plates with clear bottoms coated with poly-*D*-lysine, cultured overnight, and then incubated with a FLIPR Calcium 4 Assay Kit (Molecular Devices Corporation, USA) for 1 h at 37 °C. Fluorescence signals (λ_{EX}=488 nm, λ_{EM}=540 nm) were measured before and after the addition of $A\beta_{1-42}$ (10 μmol/L), either alone or together with LPC (10 μmol/L). To examine the source of calcium flux induced by $A\beta_{1-42}$, cells were treated with 1 μmol/L of thapsigargin (TG) for 1 h before the addition of $A\beta_{1-42}$. The total fluorescence units within 200 s were calculated.

Reduction of G2A expression in SH-SY5Y cells by small interfering RNA SH-SY5Y cells grown to 50% confluency were transfected with G2A siRNA duplexes (5′-CAACGUGUCCUUCGAAGAGtt-3′, 5′-CUCUUCGAAGGACACGUUGtt-3′) and scrambled siRNA duplexes (5′-UUCUCCGAACGUGUCACGUtt-3′, 5′-ACGUGACACGUUCGGAGAAtt-3′) (GenePharma, Shanghai, China) using the TransFast™ Transfection Reagent (Promega, CA, USA). Cells were transfected for 48 h with 3.75 μg of each siRNA according to the manufacturer's protocol (Promega, CA, USA). Efficiency of RNA interference was assessed by real-time quantitative PCR. The cultures were then treated with $A\beta_{1-42}$ (10 μmol/L) and/or LPC (0, 2, 10, and 50 μmol/L) and subjected to cell viability analyses.

Statistical analysis Data were calculated as the mean±SEM by Sigmaplot 9.0 (Systat Software Inc, USA). A Student's *t*-test was applied to determine the statistical significance. Statistical significances are shown as $^bP<0.05$ and $^cP<0.01$.

Results

Effects of LPC on $A\beta_{1-42}$-induced neuronal cell death SH-SY5Y cells were treated with $A\beta_{1-42}$ and cell viability was determined by the CCK-8 assay. Figure 1A shows that $A\beta_{1-42}$ treatment significantly reduced the viability of SH-SY5Y cells in a dose-dependent manner. Annexin V, a calcium-dependent phospholipid-binding protein, has a high affinity for phosphatidylserine (PS) and binds to PS exposed on the surface of apoptotic cells. We applied the chip-based flow cytometric assay to analyze PS externalization induced by $A\beta_{1-42}$ in SH-SY5Y cells. Annexin V-Cy5 and Calcein-AM staining showed that the number of apoptotic cells increased significantly after $A\beta_{1-42}$ treatment (Figure 1B–1D). To examine the effect of LPC on $A\beta_{1-42}$-induced cell death in SH-SY5Y cells, we treated SH-SY5Y cells with LPC at different concentrations. Our results showed that LPC inhibited the growth rate of SH-SY5Y cells in a dose-dependent manner. Furthermore, LPC greatly enhanced $A\beta_{1-42}$-induced growth inhibition (Figure 1E).

Regulation of gene expression via the $A\beta_{1-42}$ pathway To understand the molecular mechanisms involved in $A\beta_{1-42}$-induced neuronal apoptosis, we analyzed the expression of a number of apoptosis-related genes in SH-SY5Y cells with or without $A\beta_{1-42}$ treatment. First, we examined the mRNA levels of apoptosis associated genes using a quantitative PCR method. Our results showed that $A\beta_{1-42}$ increased the ratio of Bax/Bcl-x_L (Figure 2A) mRNAs and the expression of caspase-3 (Figure 2B), whereas it decreased the expression of TRAF1 in SH-SY5Y cells after $A\beta_{1-42}$ treatment (Figure 2C and 2D).

Alterations in the protein levels of Bax and caspase-3 were analyzed by Western blotting (Figure 2E) using antibodies against Bax and cleaved caspase-3 (the latter antibody recognizes the p17 large/active subunit of caspase-3). As expected, treatment with $A\beta_{1-42}$ or LPC increased Bax expression. In addition, LPC enhanced the effects of $A\beta_{1-42}$ on the expression of Bax and the p17 cleavage product of caspase-3 (Figure 2E).

To assess whether the observed increase in the cleavage

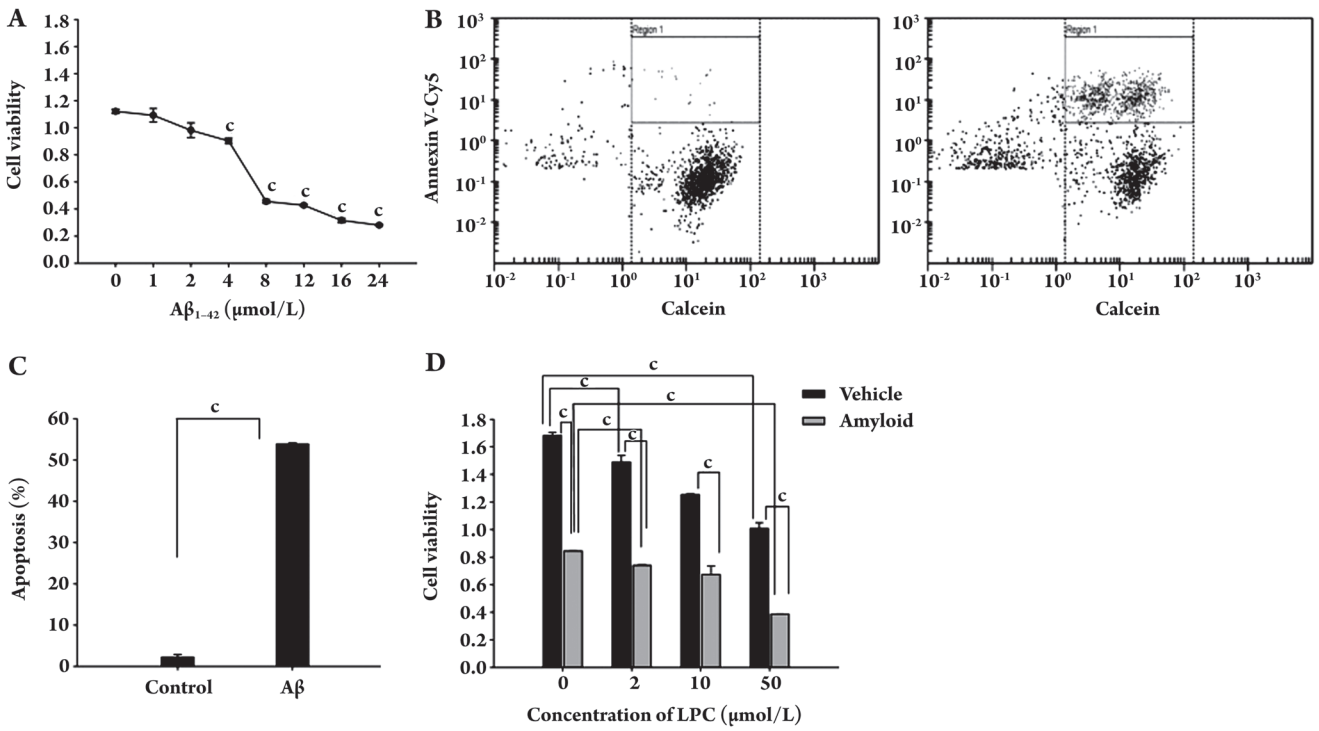

Figure 1. Effects of LPC on $A\beta_{1-42}$ neurotoxicity in SH-SY5Y cells. (A) $A\beta_{1-42}$ induced growth inhibition in a dose-dependent manner. (B–D) $A\beta_{1-42}$ mediated apoptosis in SH-SY5Y cells. (B) Apoptosis measured by Annexin V-Cy5/Calcein staining, dot plot of control SH-SY5Y cells; (C) Apoptosis measured by Annexin V-Cy5/Calcein staining, dot plot of SH-SY5Y cells after exposure to 10 μmol/L $A\beta_{1-42}$ for 48 h; (D) The percentage of Annexin V-Cy5-stained cells was calculated with respect to the total number of Calcein stained cells in a field. (E) LPC enhancement on $A\beta_{1-42}$-induced growth inhibition. SH-SY5Y cells were treated with different concentrations of LPC (0, 2, 10, and 50 μmol/L) and $A\beta_{1-42}$ (10 μmol/L) for 48 h. Cell viability was measured as described under Materials and Methods. Data are expressed as means±SEM from three independent experiments.

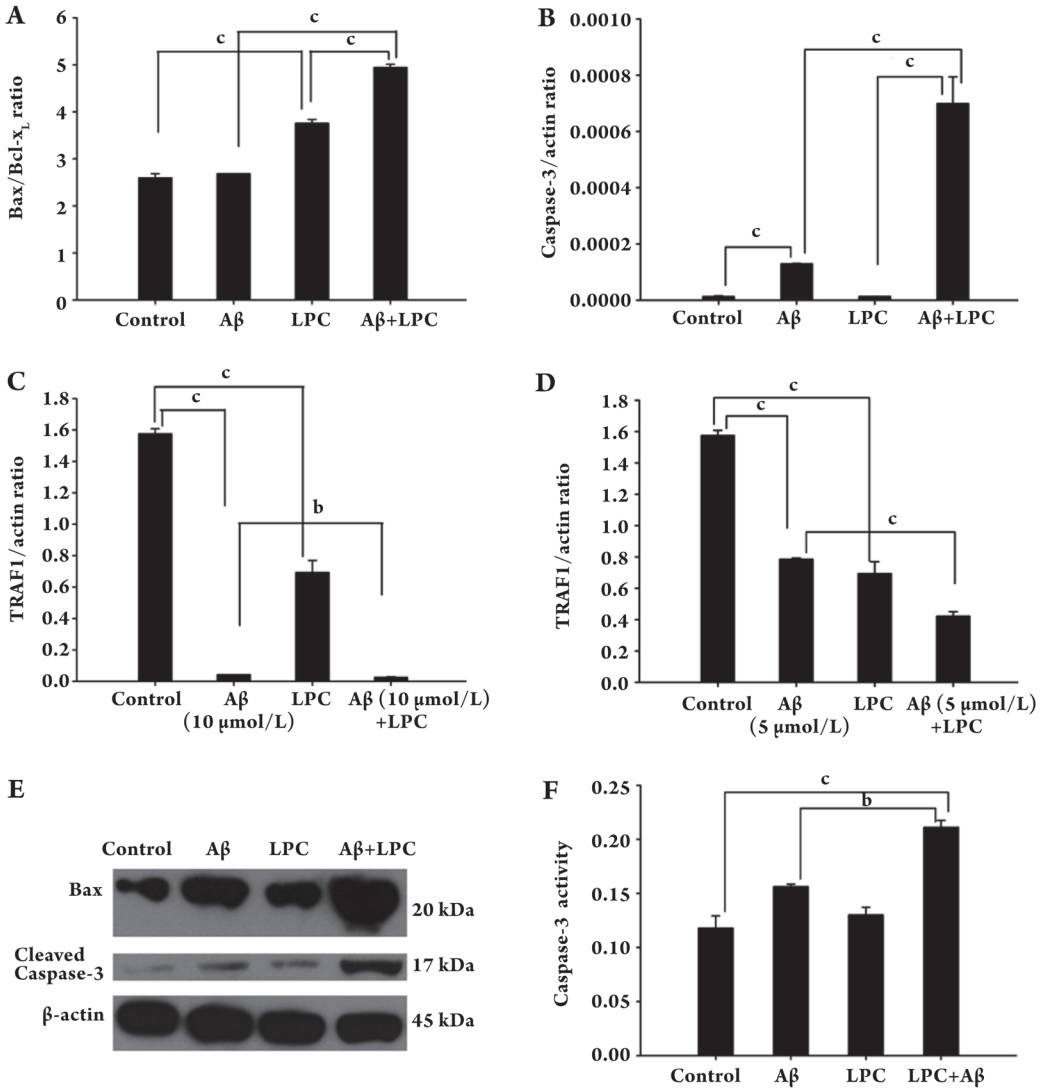

Figure 2. Effects of LPC and $A\beta_{1-42}$ on the expression of Bax, caspase-3 and TRAF1, as well as the enzymatic activity of caspase-3. SH-SY5Y cells were treated with $A\beta_{1-42}$ (5 or 10 μmol/L) and/or LPC (10 μmol/L) for 48 h. Quantitative RT-PCR analysis was performed to examine the mRNA expression of Bax, caspase-3 and TRAF1 (A–D). (E) Western blotting for determination of levels of Bax and caspase-3 using various antibodies as described under Materials and Methods. (F) caspase-3 activity in $A\beta_{1-42}$ and LPC treated SH-SY5Y cells. Caspase-3 activity was determined as described under Materials and Methods. Data were expressed as means±SEM from three independent experiments.

of caspase-3 correlated with an increase in its activity, the cleavage of Ac-DEVD-pNA, a colorimetric peptide substrate specific for caspase-3, was measured. As shown in Figure 2F, caspase-3 cleavage activity was induced in SH-SY5Y cells in the presence of Aβ, and the addition of LPC further increased this activity.

Effects of $A\beta_{1-42}$ and LPC on calcium flux Activation of a G2A receptor with LPC can induce calcium mobilization[15]. We investigated whether LPC affects $A\beta_{1-42}$-induced calcium flux in SH-SY5Y cells. Although LPC alone had no effect on calcium influx, it significantly enhanced the $A\beta_{1-42}$-induced elevation of intracellular calcium in SH-SY5Y cells (Figure 3A). We also found that $A\beta_{1-42}$ treatment alone could induce calcium influx in SH-SY5Y cells and that this activity was blocked by pretreatment of cells with 1 μmol/L of TG, a Ca^{2+}-ATPase inhibitor (Figure 3B).

Reduction of G2A inhibits the effects of LPC on $A\beta_{1-42}$-induced neurotoxicity Because LPC enhanced $A\beta_{1-42}$-induced neuronal apoptosis and was the specific ligand for an orphan GPCR, G2A, we analyzed the expres-

sion of this receptor. Our results demonstrated that LPC significantly induced expression of G2A (Figure 4A). To further examine the involvement of G2A in LPC effects on $A\beta_{1-42}$-induced neuronal apoptosis, we used siRNA to

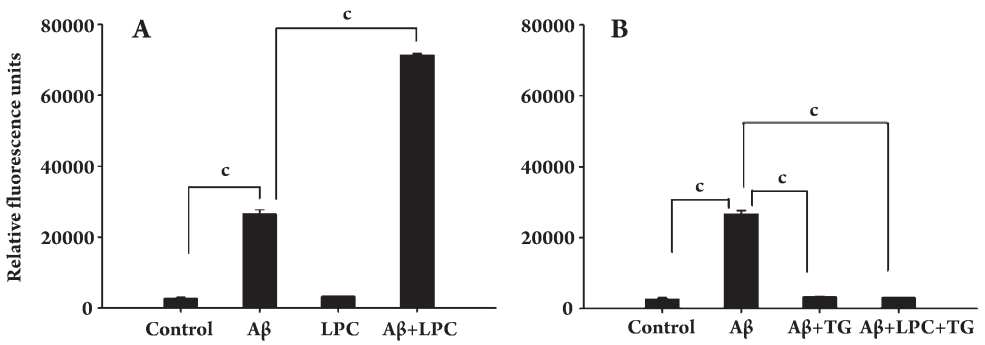

Figure 3. Effects of LPC and $A\beta_{1-42}$ on calcium levels in SH-SY5Y cells. Calcium levels were measured as described under Materials and Methods. (A) LPC (10 μmol/L) augmented the effect of $A\beta_{1-42}$ (10 μmol/L) on calcium levels. (B) After depletion of the Ca^{2+} store by the addition of TG (1 μmol/L) for 1 h, $A\beta_{1-42}$ could not change the cytosolic calcium concentration. Data were expressed as means±SEM from three independent experiments.

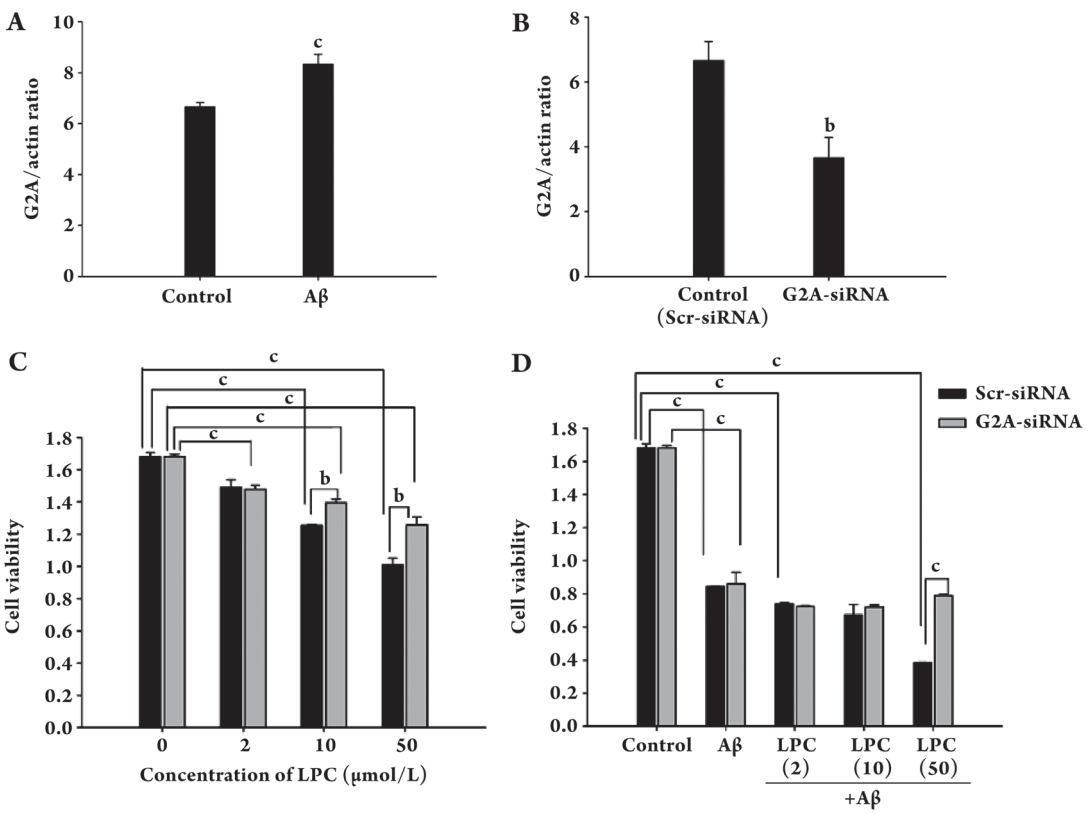

Figure 4. Reduction of G2A expression blocked LPC effects on $A\beta_{1-42}$-induced neurotoxicity. (A) G2A mRNA expression in SH-SY5Y cells after the treatment with $A\beta_{1-42}$ (10 μmol/L). (B) G2A mRNA expression in SH-SY5Y cells after transfected with siRNA specific for G2A ("G2A-siRNA") or scrambled siRNA ("Scr-siRNA") as the control. Expression of G2A was normalized to β-actin in each sample. Relative G2A expression was calculated as described under Materials and Methods. (C) The reduction of cell viability in SH-SY5Y cells exposed to LPC after transfection with siRNA. Following transfection with siRNA, the cells were incubated with different concentrations of LPC (0, 2, 10, and 50 μmol/L) for 48 h and cell viability was determined using a CCK-8 kit. (D) The cell viability in SH-SY5Y cells exposed to LPC and/or $A\beta_{1-42}$ after transfection with siRNA. Following transfection with siRNA, the cells were incubated with different concentrations of LPC (0, 2, 10, and 50 μmol/L) and $A\beta_{1-42}$ (10 μmol/L) for 48 h. Cell viability was determined using a CCK-8 kit. Data are expressed as means±SEM from three independent experiments.

reduce the expression of the orphan GPCR. Transfection of SH-SY5Y cells with G2A-specific siRNA resulted in a 55% inhibition of G2A expression (Figure 4B). Furthermore, the down-regulation of G2A expression reduced the effects of LPC on Aβ-induced neurotoxicity (Figures 4C and 4D).

Discussion

Aβ peptide, the main component of senile plaque, plays a central role in AD pathogenesis[17]. Oxidative stress increased the production of Aβ peptides in neuronal cell lines and in AD animal models[18–20]. Genetic and biochemical analysis has demonstrated that Aβ may be a causative factor both in neuronal apoptosis and in the cognitive impairments seen in AD patients[21, 22]. Furthermore, much recent evidence indicates that Aβ may act as an initiating factor, inducing specific signaling pathways leading to neuronal apoptosis[23–28]. However, the molecular targets of Aβ effects remain unidentified.

Lipid peroxidation is a predominant manifestation of oxidative stress in the central nervous system (CNS)[16]. Accumulating data suggests that increased lipid peroxidation is an early event in the pathogenesis of AD. In this study, we used SH-SY5Y cells as a cellular model to examine the molecular basis of $A\beta_{1-42}$-induced neuronal apoptosis and to address a possible role for lipid peroxidation in amyloid neurotoxicity. For the latter, we examined the effect of LPC, a product of lipid peroxidation, on $A\beta_{1-42}$-induced apoptosis. As reported, $A\beta_{1-42}$ decreased cell viability in a dose-dependent manner and effectively induced apoptosis in SH-SY5Y cells. We further found that $A\beta_{1-42}$ regulated the expression of a number of apoptosis associated genes and that treatment of SH-SY5Y cells with LPC not only inhibited the proliferation of SH-SY5Y cells, but also enhanced $A\beta_{1-42}$-induced neuronal apoptosis.

Apoptosis is a tightly regulated process involving changes in the expression of a distinct set of genes[29]. A tumor necrosis factor death receptor (TNF) signaling cascade is required for Aβ-induced neuronal death[30]. TNF receptor-associated factor 1 (TRAF1) participates in the inhibition of apoptosis, possibly via recruitment of anti-apoptotic factors, such as cIAP1 and cIAP2, into death receptor signaling complexes[31]. Interestingly, we found that both $A\beta_{1-42}$ and LPC could decrease the expression of TRAF1 in SH-SY5Y cells and that LPC enhanced the effect of $A\beta_{1-42}$ on TRAF1 mRNA expression. Caspases, including caspase-1[32], caspase-2[23], caspase-3[33], caspase-8[34], and caspase-12[24], are involved in the pathogenesis of Aβ toxicity *in vitro*. Because caspase-3 is a critical downstream protease in the apoptotic cascade, we examined its expression in SH-SY5Y cells after $A\beta_{1-42}$ treatment. Our results demonstrated that $A\beta_{1-42}$ significantly increased the expression of caspase-3. Neuronal apoptosis induced by Aβ is related to Bax, a proapoptotic Bcl-2 family member, or Bcl-x_L, an antiapoptotic analogue[35, 36]. $A\beta_{1-42}$ also increased the mitochondrial ratio of Bax to Bcl-x_L in SH-SY5Y cells, and LPC augmented the $A\beta_{1-42}$-induced elevation of caspase-3 and elevated Bax/Bcl-x_L ratio at both the mRNA and the protein levels. In agreement with the expression analysis results, LPC also increased the effect of $A\beta_{1-42}$ on the enzymatic activity of caspase-3.

One possible explanation for the effects of LPC on neuronal apoptosis is that LPC binds to its specific receptor and performs its physiological and pathological functions via a downstream second messenger system. Many studies have demonstrated that LPC is a high-affinity ligand for G2A[15]. Interestingly, we found that $A\beta_{1-42}$ treatment significantly induced the expression of G2A in SH-SY5Y cells. Deregulation of intracellular calcium signaling is implicated in the pathogenesis of Alzheimer's disease[37]. The activation of a G2A receptor through LPC could induce calcium mobilization[15]. Indeed, our observation that the elevation in intracellular calcium levels caused by $A\beta_{1-42}$ could be blocked by TG suggests that the elevation of calcium levels was from intracellular calcium stores. Our results further showed that LPC treatment alone had no effect on intracellular calcium concentration, but could enhance the elevation of $A\beta_{1-42}$-induced calcium concentration in SH-SY5Y cells. Furthermore, our observation that G2A siRNA blocks the LPC effect suggests that the orphan GPCR may be involved in calcium elevation and $A\beta_{1-42}$-induced neurotoxicity. These results provide evidence that LPC may exert its neurotoxic effects through orphan GPCR signal pathways. We propose further studies to help elucidate the roles of G2A in neurodegenerative phenotypes in the AD brain.

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Author contribution

Ying-he HU and Zhen-xia QIN designed research and wrote the paper; Zhen-xia QIN performed research and analyzed data; Hui-yan ZHU contributed new analytical tools and reagents.

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