Protective Effects of Alpha-Lipoic Acid on Glutamate-Induced Cytotoxicity in C6 Glioma Cells

Euteum Park, Jungsoo Gim, Do Kyung Kim, Chun-Sung Kim, and Hong Sung Chun

Department of Biomedical Science, Chosun University; Gwangju 61452, Republic of Korea: Department of Oral Physiology, College of Dentistry, Chosun University; Gwangju 61452, Republic of Korea: Department of Oral Biochemistry, College of Dentistry, Chosun University; Gwangju 61452, Republic of Korea.

Received August 7, 2018; accepted October 23, 2018

Glutamate-mediated cytotoxicity has been implicated in the pathogenesis of neurological diseases, including Parkinson’s disease, Alzheimer’s disease, and stroke. In this study, we investigated the protective effects of alpha-lipoic acid (ALA), a naturally occurring thiol antioxidant, on glutamate-induced cytotoxicity in cultured C6 astroglial cells. Exposure to high-dose glutamate (10 mM) caused oxidative stress and mitochondrial dysfunction through the elevation of reactive oxygen species, depletion of glutathione, and loss of the mitochondrial membrane potential (ΔΨm). Pretreatment with ALA (200 µM), however, significantly inhibited the glutamate-induced oxidative stress and mitochondrial dysfunction. ALA pretreatment dose-dependently suppressed glutamate-induced apoptotic events including altered nuclear morphology and activation of caspase-3. In addition, ALA significantly attenuated glutamate-induced endoplasmic reticulum (ER) stress markers; namely, glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF6), protein kinase regulated by RNA (PKR)-like ER-associated kinase (PERK), eukaryotic translation initiation factor 2 alpha (eIF2α), inositol-requiring enzyme 1 (IRE1), CCAAT/enhancer binding protein homologous protein (CHOP), and caspase-12. We confirmed that CHOP and caspase-12 are key mediators of glutamate-induced ER stress. Furthermore, exposure of the cells to a caspase-12-specific inhibitor and CHOP small interfering RNAs (siRNAs) led to restoration of the ΔΨm that was damaged by glutamate treatment. These results suggest that ALA can effectively suppress oxidative stress, mitochondrial dysfunction, and ER stress in astroglial cells.

Key words alpha-lipoic acid; apoptosis; glutamate; mitochondrial dysfunction

INTRODUCTION

Glutamate, an amino acid, is the most abundant excitatory neurotransmitter in the central nervous system. Although glutamate stimulates ionotropic and/or metabotropic glutamate receptors and has an important role in normal neural physiological processes, its excessive amount can induce excitotoxicity, thereby elevating the neuronal damage in many neurological diseases.1,2 Glutamate toxicity has been demonstrated in various types of cell lines including neuronal, oligodendroglial, astroglial, and retinal ganglion cells.3 Several studies have suggested that glutamate cytotoxicity induces the elevation of intracellular calcium levels and—following the production of reactive oxygen species (ROS)—mitochondrial dysfunction and endoplasmic reticulum (ER) stress.4,5

Aside from excitotoxicity, excessive glutamate can also lead to cystine/glutamate antiporter inhibition-related oxidative stress. The excitotoxicity mechanism is associated with over-activation of the G-protein-coupled metabotropic glutamate receptor and ionotropic glutamate receptors, such as those for N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainic acid.6 Prolonged excitotoxicity increases the intracellular calcium concentration and potentiates the accumulation of ROS.7 In addition, excessive glutamate can lead to reverse action of the cystine/glutamate antiporter, where the inhibition of cysteine uptake results in the depletion of glutathione (GSH, an endogenous antioxidant) and subsequent increased stimulation of ROS.8 For these reasons, much interest has been focused on antioxidants that can rescue neurological diseases involving glutamate cytotoxicity.

Alpha-lipoic acid (ALA), a naturally occurring thiol antioxidant, has been used in the treatment of diabetes and cardiovascular disease.9,10 In addition, previous studies have suggested that ALA can easily cross the blood–brain barrier and effectively protect the central nervous system.11,12 Oxidative stress-mediated pathways can affect cellular organelles, including the mitochondria and ER. Through its potent antioxidant properties, ALA improved mitochondrial function in dorsal root ganglion sensory neurons and skin fibroblast.13,14 Moreover, ALA has been shown to attenuate ER stress in human aortic endothelial cells as well as in murine liver and thyroid cells.15,16

Astroglial cells are known to be able to support and promote neuronal survival, and therefore the damage of these cells may increase neuronal loss. In particular, excessive extracellular glutamate impairs not only neurons but also astroglial cells.18 C6 glioma cells have been widely used as an astroglial cell model to study their functions and gliotoxin-mediated signaling pathways. Previous studies have revealed that glutamate can induce cytotoxicity in C6 cells.19,20 Recent papers showed that ALA can protect C6 cells from ammonia toxicity, and it also intrinsically increased the glutamate uptake, glutamine synthetase activity, and GSH content in C6 cells.21,22 However, the detailed mechanisms underlying the protective effects of ALA against excessive glutamate in astroglial cells are not fully understood. In this study,

*These authors contributed equally to this work.

© 2019 The Pharmaceutical Society of Japan
therefore, we explored whether ALA can attenuate both ER stress and mitochondrial dysfunction in glutamate-treated C6 astroglial cells, and obtained evidence on the interplay between the mitochondria and ER and its modulation of astroglial cell death.

MATERIALS AND METHODS

**Chemicals and Treatments** ALA, glutamate, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Taq polymerase and M-MLV reverse transcriptase were purchased from iNTRON Biotechnology Inc. (Seongnam, Korea). Antibodies against protein kinase regulated by RNA-like ER-associated kinase (PERK), phosphorylated PERK (p-PERK), eukaryotic translation initiation factor 2 alpha (eIF2α), phosphorylated eIF2α (p-eIF2α), inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), glucose-regulated protein (GRP78/BiP), CCAAT enhancer binding protein homologous protein (CHOP/GADD153), caspase-12, and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The phosphorylated IRE1 (p-IRE1) antibody was obtained from Abcam (Cambridge, U.K.). The cell culture medium and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, U.S.A.). The C6 cells were cultured and maintained at 37°C in Dulbecco’s modified Eagle’s medium containing 5% FBS and penicillin (100 U/mL)–streptomycin (100 µg/mL). Cells were seeded at 7 × 10^4 cells/mL into the medium and incubated for 24 h, following which they were pretreated with ALA for 1 h before glutamate treatment. To prevent any serum effect and direct interaction between the treated chemicals, the culture medium was changed to a serum-free one before the treatments were carried out. Cell viability was assessed using the PrestoBlue assay (Invitrogen), which monitors the reduction of resazurin into fluorescent resorufin. In brief, 10 µL of 10× PrestoBlue reagents was added to cultured cells in each well of a 96-well plate, which was then incubated at 37°C for 1 h. The absorbance was determined at 560 nm (excitation)/590 nm (emission) using a fluorescence multi-well plate reader (Tecan Infinite F200; Tecan, Grödig, Austria).

**Evaluation of ROS Production** Intracellular ROS production was evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) as the probe. The non-fluorescent H2DCF-DA is oxidized to the highly fluorescent dichlorofluorescein (DCF) by cellular ROS. In brief, C6 cells were treated with glutamate (10 mM) with or without ALA for up to 24 h. After washing with Hank’s balanced salt solution (HBSS), the cells were incubated with H2DCF-DA (10 µM) for 30 min and then rinsed twice with HBSS. The fluorescence of DCF was determined at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The relative fluorescence intensity was expressed as a percentage.

**Mitochondrial Membrane Potential Assay** The mitochondrial membrane potential (ΔΨm) was measured using JC-1, a mitochondrion-specific lipophilic cationic fluorescent dye. JC-1 forms red fluorescent J-aggregates under normal cell conditions, but exists preferentially in a green fluorescent monomeric form under cell damaged conditions. Thus, the red-to-green ratio correlates with ΔΨm. For this assay, C6 cells were treated with glutamate (10 mM) for 24 h with or without ALA (200 µM) and then incubated with JC-1 (5 µg/mL) for 15 min at 37°C in the dark. After washing three times with phosphate-buffered saline (PBS), the fluorescence intensity was measured with a fluorescence plate reader (red: 535 nm excitation/590 nm emission; green: 485 nm excitation/535 nm emission). The result was determined as the ratio of red-to-green fluorescence and presented as a percentage relative to the untreated control.

**Assay for Glutathione (GSH) Content** Cellular GSH levels were assessed using the cell permeant fluorescent dye monochlorobimane, which has exclusively high affinity for GSH. C6 cells were pretreated with ALA (200 µM) for 1 h and then treated with glutamate (10 mM) for 24 h in black-6 well culture plates. After washing with HBSS, the cells were incubated with monochlorobimane (40 µM) for 20 min in the dark. The cells were then rinsed twice with HBSS and their fluorescence intensity was measured at excitation and emission wavelengths of 360 and 465 nm, respectively. The GSH levels were calculated on the basis of a known amount of glutathione and expressed as a relative percentage of untreated control samples.

**Assessment of Apoptosis and Necrosis** To detect and distinguish apoptotic cells and necrotic cells, nuclear staining with Hoechst dye 33342 and propidium iodide (PI) was performed. C6 cells were treated with glutamate (10 mM) for 24 h with or without pretreatment with ALA and fixed with 1% paraformaldehyde (in PBS) for 30 min at room temperature. After fixation, the cells were washed with PBS twice and then stained with Hoechst 33342 (10 µM) for 10 min and further stained with PI (10 µM) for 10 min in dark. Stained cells were washed with PBS three times and examined by fluorescence microscopy (IX-71, Olympus, Tokyo, Japan). The apoptotic cells were determined as bright condensed and fragmented nuclei and calculated as the ratio of apoptotic nuclei to the total number of nuclei. PI positive cells with pink to red color were counted as necrotic cells. At least five randomly chosen fields were counted in a given experiments.

**Measurement of the Activation of Caspase-3** Caspase-3 activity was determined to evaluate apoptotic cell death. For the colorimetric measurement of caspase-3 activity, cells were scraped from the plate with cold lysis buffer (50 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% CHAPS) and incubated for 5 min on ice and then clarified by centrifuging at 10000 × g for 4°C for 10 min. Prepared protein samples (each 10 µg) were incubated with caspase-3 substrate (200 µM of AC-DEVD-pNA; Sigma-Aldrich) at 25°C. Formation of p-nitroaniline (pNA) from the reaction was measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, U.S.A.) with 405 nm wavelength over 1 h period. Activity was expressed as pmol substrate hydrolyzed/min.

**Western Blot Analysis** The differential expression of various proteins was determined by Western blot analysis. After treatment, the C6 cells were washed with PBS and lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate in PBS). The lysates were passed through a 21-gauge needle (to reduce the viscosity of and shear the nucleic acids) and then incubated on ice for 30 min. After centrifugation at 14000 × g for 10 min at 4°C, the supernatant...
was collected as the whole protein lysate and a 30-µg sample was separated by SDS-polyacrylamide gel electrophoresis (PAGE). The protein bands on the gel were transferred to a polyvinylidene difluoride membrane, which was subsequently blocked for 2 h with TBST buffer containing 5% non-fat dry milk (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20, and 5% non-fat dry milk) at room temperature. After washing in TBST buffer without the non-fat dry milk, the membrane was reacted overnight with primary antibodies for p-PERK, p-eIF2α, p-IRE1, ATF6, GRP78, CHOP, caspase-12 (1 : 1000 dilution), or actin (1 : 4000 dilution) at 4°C, and then with horseradish peroxidase-conjugated secondary antibodies (1 : 2000 dilution) for 2 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Pittsburgh, PA, U.S.A.).

Small Interfering RNA (siRNA) Experiments Two different non-overlapping CHOP siRNAs and an irrelevant negative control siRNA were designed and produced by Bioneer Inc. (Daejeon, Korea). The two rat CHOP gene sequences used were siCHOP-1 (sense: 5'-GGA AGA ACUAGG AAA CGG Att-3'; antisense: 5'-UCGGUUUCCAGUUCUUCt3') and siCHOP-2 (sense: 5'-CUUGG AAA CAGCGCAUGAt3'; antisense: 5'-UUCAGCGCUGUUCCAGt3'). Transfection of the C6 cells with the siRNA duplex was performed with TransIT-TKO transfection reagent (Mirus Bio LLC, Madison, WI, U.S.A.). After 48 h transfection, the cells were treated with glutamate (10 mM) or the cell viability and ΔΨm were measured. The silencing effect was confirmed by Western blot assay.

Statistical Analysis All data were expressed as the mean ± standard error of the mean (S.E.M.) and analyzed using the SPSS 12.0 software package (SPSS Inc., Chicago, IL, U.S.A.). A one-sample t-test was used for comparisons between two groups. Differences between individual groups were examined by one-way factorial ANOVA, followed by Duncan’s post hoc test. The statistics were evaluated at the significance level of 2α = 0.01.

RESULTS Protective Effect of ALA against Glutamate-Induced Cytotoxicity C6 glioma cells are derived from rat brain and have been widely used as an astroglial cell model because this cell line expresses normal astroglial markers and shows functional analogy to normal astroglial cells. In this study, C6 cells were adopted as an astroglial cell model to investigate the effects of ALA on glutamate-mediated cytotoxicity. First, we evaluated the cytotoxic response of C6 cells to various concentrations of glutamate. As shown in Fig. 1A, glutamate treatment for 24 h induced progressive cell death in a concentration-dependent manner. Under our experimental conditions, 10 mM glutamate induced approximately 40% cell death and therefore this concentration was used in subsequent experiments. Next, we investigated the effect of ALA on glutamate-induced C6 cell death. As shown in Fig. 1B, ALA at 50–400 µM did not intrinsically affect the viability of C6 cells.
ALA Inhibited Glutamate-Induced Total ROS Production and Restored the Glutamate-Induced Mitochondrial Dysfunction and Cellular GSH Content Depletion

Excessive glutamate has been known to stimulate ROS generation and mitochondrial disturbance. To investigate the changes of ROS generation in C6 cells, we measured the intracellular levels using the fluorescent dye H$_2$DCF-DA. Cells treated with glutamate (10 mM) alone for various time periods (0.5–24 h) showed increased intracellular ROS in a time-dependent manner until 24 h and peaked at 12 h after treatment (increased by 2.7-fold at 12 h and 2.5-fold at 24 h). However, ALA pretreatment effectively attenuated the glutamate-induced ROS increase in the cells (Fig. 1C).

It has been suggested that mitochondrial dysfunction with a loss of ΔΨ$_{m}$ integrity is closely linked with ROS generation and cell death. To evaluate ΔΨ$_{m}$ in the C6 cells, the mitochondrial-specific fluorescent dye JC-1 was used. As shown in Fig. 1D, untreated control cells and cells treated with ALA (200 µM) alone showed an intact ΔΨ$_{m}$. However, treatment of C6 cells with glutamate (10 mM) for 24 h drastically reduced the ΔΨ$_{m}$ (41.26% of control). Notably, the glutamate-induced ΔΨ$_{m}$ loss was significantly prevented by ALA pretreatment (86.91% of control).

The representative antioxidant GSH has been known to protect astrocytes from oxidative stress damage. To ascertain the anti-oxidative effect of ALA against glutamate, we measured the intracellular GSH levels (Fig. 1D). Glutamate significantly reduced the GSH levels to 62.68% compared with that in the untreated control cells. However, pretreatment with ALA markedly restored the GSH to a normal level (99.56% of control).

ALA Ameliorated Glutamate-Induced Apoptosis and Caspase-3 Activation

We investigated the effect of ALA on glutamate-induced apoptotic characteristics, such as nuclear morphology changes and caspase-3 activation in C6 cells. A significant proportion of glutamate-induced cell death was apoptotic, based on double staining with Hoechst 33342 and PI. We observed a significant increase in condensed, fragmented nuclei after 24 h treatment with glutamate (10 mM). However, a low percentage of nuclei were stained pink to red by the necrotic marker dye PI (Fig. 2A). Moreover, we found...
that ALA pretreatment dose dependently inhibited glutamate-induced apoptotic nuclear damages (Fig. 2A). As quantified in Fig. 2B, although the level of apoptotic nuclei in the glutamate treated cells was 30.76 ± 2.19%, a statistically significant reduced apoptotic rate was detected when cells were pretreated with ALA 50 µM (20.73 ± 2.53%), 100 µM (6.59 ± 0.73%), and 200 µM (3.52 ± 0.91%), respectively. The nuclear morphology was intact with minimal apoptotic rate in both untreated control cells (1.28 ± 0.34%) and ALA alone treated cells (1.39 ± 0.49%).

Next, we examined caspase-3 activity as another representative apoptotic marker. A shown in Fig. 2C, the exposure of C6 cells to 10 mM glutamate for various time points (6, 12, 24 and 48 h) increased caspase-3 activity by 1.22-, 2.05-, 3.91- and 3.65-fold, respectively. In contrast, ALA (50–200 µM) pretreatment strongly attenuated the effects of glutamate on caspase-3 activity at each time point dose dependently. These results positively verify that the protective effect of ALA is mediated by anti-apoptotic pathway at least in part.

Effects of ALA on the Expression of ER Stress Markers

To investigate the involvement of the ER stress pathway in glutamate-induced cell death and ALA-mediated protection, we analyzed the expressions of ER stress biomarkers by Western blot assay. Glutamate (10 mM) treatment for 6–48 h time-dependently changed the levels of ER stress markers (Fig. 3A). The level of phosphorylated PERK, phosphorylated eIF2α, phosphorylated IRE1, cleaved caspase-12, and GRP78 was assayed by Western blot analysis. Actin expression was shown as a loading control. The intensity of each band was measured by densitometric analysis and the normalized quantification values were written below each band. The experiments were repeated three times with similar results. (B, C) Cells were pretreated with ALA (200 µM) for 1 h prior to treatment with glutamate (10 mM) for 24 h. The protein expression was analyzed by Western blotting. At the same conditions, actin expression was determined for normalization. The specific expression levels were quantified by densitometric analysis and assessed as the ratio against the value of the untreated control in each group. Data are expressed as the mean ± S.E.M. from three independent experiments. * Statistically significant as compared with the untreated control group. ^ Statistically significant as compared with the glutamate-alone treatment.

Fig. 3. Effects of Alpha-Lipoic Acid (ALA) on the Protein Levels of Endoplasmic Reticulum (ER) Stress Markers in Glutamate-Treated C6 Cells

(A) C6 cells were treated with 10 mM glutamate for the indicated time periods. The change in protein expression levels of the ER stress markers, phosphorylated PERK (p-PERK), phosphorylated eIF2α (p-eIF2α), phosphorylated IRE1 (p-IRE1), pro-ATF6, CHOP, cleaved caspase-12, and GRP78 was assayed by Western blot analysis. Actin expression was shown as a loading control. The intensity of each band was measured by densitometric analysis and the normalized quantification values were written below each band. The experiments were repeated three times with similar results. (B, C) Cells were pretreated with ALA (200 µM) for 1 h prior to treatment with glutamate (10 mM) for 24 h. The protein expression was analyzed by Western blotting. At the same conditions, actin expression was determined for normalization. The specific expression levels were quantified by densitometric analysis and assessed as the ratio against the value of the untreated control in each group. Data are expressed as the mean ± S.E.M. from three independent experiments. * Statistically significant as compared with the untreated control group. ^ Statistically significant as compared with the glutamate-alone treatment.

To further investigate the potential anti-ER stress effects of ALA, we examined the expression levels of other ER stress response biomarkers (viz., CHOP and caspase-12). We observed significantly increased protein expression of CHOP (5.27-fold) by glutamate, which was also markedly inhibited by ALA (Figs. 3B, C). To evaluate the contribution of caspase-12 to glutamate-induced ER stress, we determined its cleavage (i.e., activation) in C6 cells. Treatment with 10 mM...
glutamate markedly induced the cleavage of caspase-12, whereas pretreatment of cells with 200 μM ALA effectively attenuated this effect (Figs. 3B, C).

Role of CHOP and Caspase-12 in Glutamate-Induced Cytotoxicity and Mitochondrial Dysfunction Next, we examined whether CHOP induction is critical for glutamate-mediated C6 cell death. Upon the silencing of CHOP expression by specific siRNAs, glutamate-induced C6 cell death was dramatically attenuated (Fig. 4B). To further confirm that activation of caspase-12 is required for glutamate-induced cell death, C6 cells were pretreated with a specific caspase-12 inhibitor, Z-ATAD-fluoromethylketone (Z-ATAD-FMK; 0.01–1 μM), for 1 h, and then stimulated with glutamate (10 mM) for 24 h. As shown in Fig. 4B, pretreatment with Z-ATAD-FMK increased the cells’ resistance to glutamate-induced death. Of interest, the combined treatment with CHOP siRNA and caspase-12 inhibitor induced a boosted protective effect.

We also determined whether mitochondrial dysfunction and ER stress are linked in glutamate-mediated glial cell death. As shown in Fig. 4C, glutamate-induced ΔΨm reduction was moderately reversed by the caspase-12 inhibitor and effectively restored by the CHOP siRNAs. In addition, there is some additive effect of caspase-12 inhibitor and CHOP siRNA co-treatment on the maintenance of mitochondria function. These results suggest that mitochondrial damage and ER stress pathways together mediate glutamate-induced C6 cell death, and ALA effectively attenuates those pathways to protect the astroglial cells.

DISCUSSION

The present study suggested that ALA can inhibit glutamate-induced apoptotic cell death in C6 cells. ALA, a naturally occurring thiol antioxidant, can easily pass through the biological membrane and blood–brain barrier (BBB), chelate transition metals, and recycles other endogenous antioxidants such as GSH, vitamin C, and other vitamins.28,29 Because of these beneficial effects, ALA has been applied as a therapeutic agent for treating various diseases, including neurological disorders.9–12

C6 rat glioma cells strongly express glial fibrillary acidic protein (GFAP) and S100B (unique astrocyte marker proteins). In addition, C6 cells express both ionotropic and metabotropic glutamate receptors.3 Therefore, C6 cells are widely used to as a tool for studying the function of astroglial cells and excitotoxicity mechanism. Excessive glutamate triggers excitotoxicity on C6 cells in a dose and time dependent manner. Its toxic concentration varies between 0.01 and 20 mM according to experimental culture conditions.3,19 In this study, 10 mM glutamate was used as a neurotoxicant because sustained high-dose glutamate induces neuronal damage as well as astroglial cell death accompanied with functional exhaustion and glial swelling.18,30 Although a major function of astrocytes is the uptake and buffering of excessive extracellular glutamate to protect neighboring neurons from glutamate excitotoxicity, excessive glutamate can elevates astrocyll cell death and consequently overall neurodegeneration.2,30 It has been suggested that astrocytes play dual roles as protector to prevent surrounding neuron’s damage while at the same time affect neurons and contribute to variety of neurological diseases when malfunctioned by neurotoxics.32

Several studies have suggested that glutamate elevates cellular calcium and ROS levels, leading to mitochondrial dysfunction and ER stress.4,5,33 Previous studies have demonstrated that the glutamate-induced elevation of intracellular ROS and oxidative stress was attributable to a depletion of the endogenous antioxidant GSH.4,34,35 We also observed that glutamate induced ROS elevation and mitochondrial dysfunction together with GSH depletion in C6 cells (Fig. 1). However, ALA pretreatment effectively suppressed those oxidative stress-related changes, indicating a protective effect of ALA is due to antioxidative properties. GSH exists ubiquitously in most cells and exerts critical protective effects. Under normal
conditions, GSH is synthesized in the cytosol and properly maintained in the mitochondria after transported, but neurotoxicants induce depletion of GSH and subsequent ROS increase and mitochondrial damage. It has been proposed that depletion of cellular GSH is associated with apoptosis. Although overexpression of anti-apoptotic molecules elevated the level of GSH, pro-apoptotic molecules significantly down-regulated GSH contents.

We showed that glutamate increased apoptotic events in C6 cells and ALA dose-dependently prevented those events (Fig. 2). When our results were analyzed in detail, glutamate (10 mM) treatment induced approximately 40% cell death, but only 30.76% apoptosis rate and 2.42% necrosis rate were detected (Fig. 2B). Even though apoptosis is the major event in C6 cell death by glutamate, it is likely that there is other types of cell death, such as autophagy and cornification.

The ER exists in all eukaryotes and plays a major role in protein synthesis and proper folding. However, various pathological stimuli can impair the normal function of the ER and accumulate unfolded/denatured proteins in the organelle, with dysregulation of calcium homeostasis. These dysfunctions are collectively called ER stress and lead to the pathogenesis of neurological diseases. ER stress initiates an adaptive mechanism called the unfolded protein response, which elevates ER chaperones to decrease the misfolded or unfolded proteins. However, persistent and severe ER stress will trigger the apoptotic pathway.

The paradoxical ER stress pathways are initiated by three ER transmembrane proteins (i.e., ER stress sensors): PERK, IRE1, and ATF6. Under ER stress conditions, the three ER stress sensors depart from GRP78 (BiP), a chaperone in the ER lumen, and are activated by phosphorylation (PERK and IRE1) or by cleavage and translocation to the nucleus (ATF6). The activated PERK subsequently phosphorylates eIF2α, which attenuates protein synthesis to reduce the ER overload, or induces ATF4 to stimulate the cell death pathway. The activated IRE1 cleaves the mRNA of the transcription factor XBP1 in the cytosol, which can then translocate to the nucleus to regulate downstream genes related to ER stress suppression or apoptotic cell death induction.

Several studies have demonstrated that glutamate induces ER stress pathways during cell death in various cell types including C6 cells. In addition, recent studies suggest that ALA can inhibit the ER stress in various cells such as thyroid cells and aortic endothelial cells. However, the attenuation of the ER stress by ALA in astroglial cells has not been reported until now. Our results revealed that ALA strongly attenuated the ER stress markers which were induced by glutamate in C6 cells (Fig. 3). To the best of our knowledge, this is the first report on the suppressive effects of ALA against glutamate-mediated ER stress in astroglial cells.

Accumulating evidence has suggested that CHOP and caspase-12 are crucial molecules in ER-stress-mediated cell apoptosis. Although CHOP is expressed at low levels under normal conditions, it is strongly induced by severe and prolonged ER stress. In addition, the ER membrane-bound caspase-12 is cleaved and activated by ER stress, and then induces the downstream death molecule caspase-3. Our results clearly showed that ALA can prevent glutamate-stimulated CHOP induction and caspase-12 activation. In addition, we provided critical evidence that these two ER stress-related molecules are key contributors to glutamate-mediated C6 astroglial cell death (Fig. 4B).

Because glutamate induced both mitochondrion-related oxidative stress and ER stress in C6 cells, we speculated on the interplay between the ER and mitochondria in our experiment conditions. The ER and mitochondria are physically connected through the ER-mitochondria juxtagapositions called mitochondria-associated ER membranes (MAMs). These specialized regions are crucial to regulate Ca2+ dynamics and ROS-mediated signals between the ER and the mitochondria. Recent data suggest that mitochondrial dysfunction caused ATP depletion and subsequent ER disturbance in oligodendroglia. Adversely, several studies proposed that ER stress induced mitochondrion-dependent apoptotic pathways. Accumulated ER stress triggers the translocation of Bax and Bad to mitochondria with collapse of ΔΨm, and then the mitochondria release the cytochrome c which is related to the apoptotic signaling pathways. It has been demonstrated that ER stress and mitochondrial dysfunction are closely linked events. Our results revealed that the inhibition of key ER stress molecules (CHOP and caspase-12) can suppress mitochondrial dysfunction (Fig. 4C). Although this is not enough evidence to prove the direct interplay between the ER and mitochondria, our results suggest that ER stress can sensitize mitochondria to evoke astroglial cell death.

Accumulating evidences suggest that astrocyte dysfunction is closely linked to several neurological diseases, such as Alzheimer’s disease, Parkinson’s disease, and ischemic stroke. Although those neurological diseases show complex pathogenesis, both astrocytes and neurons are commonly affected in various diseases. In normal conditions, astrocytes provide structural and functional support for neurons. Astrocytes transfer substrates for energy, neurotransmitters, and antioxidants to neurons. Also, astrocytes maintain the extracellular environment of central nervous system by uptake glutamate, K+ ions, and lactate. Interestingly, astrocytes become reactive in the disease state, characterized by the hypertrophy and proliferation around the injured or pathological regions. The rapidly expanding astrocytes following stroke or other neurological damage forms glial scar which has a role as surrounding walls to protect and maintain injured region. Although the formation of glial scar may be beneficial in restricted area of damage for a while, it serves as a negative barrier to long-term restoration. Despite astrocytes respond to reduce the neurological functions in the early stages of the disease, deleterious etiological causes impair astrocytic functions inducing increased extracellular glutamate-mediated excitotoxicity, oxidative stress, and following cell death.

Recent report suggested that ALA attenuated the astrocytes reactivity and formation of glial scar, and restored the BBB in trauma brain injury. Regarding as a potent antioxidant, ALA reduced damage from ischemia–reperfusion, Alzheimer’s disease, and Parkinson’s disease, which are closely related to oxidative stress under pathological circumstances. Thus, ALA may be a good candidate therapeutic compound, which can open up a novel strategy for neurological diseases that correlate with astrocyte dysfunction.

CONCLUSION

Our present study suggested that ALA can protect C6 astro-
glial cells from glutamate-mediated apoptosis. The protective effects of ALA were mediated through the attenuation of ROS production, depletion of GSH, decrease in mitochondria function, and elevation of ER stress. The results also suggested that ER stress induced further Δψm loss during glutamate-mediated C6 astroglial cell death. These findings support a new clinical strategy of using ALA for various neurological diseases that are correlated with glutamate-mediated astroglial cell damage.

Acknowledgment This study was supported by research fund from Chosun University, 2017.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Wang Y, Qin ZH. Molecular and cellular mechanisms of excitotoxic neuronal death. Apoptosis, 15, 1382–1402 (2010).
2) Lai TW, Zhang S, Wang YT. Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog. Neurobiol., 115, 157–188 (2014).
3) Kritis AA, Stamoula EG, Paniskaki KA, Vavilis TD. Researching glutamate-activated cytotoxicity in different cell lines: a comparative/collective analysis/study. Front. Cell. Neurosci., 9, 91 (2015).
4) Greenwood SM, Connolly CN. Dendritic and mitochondrial changes during glutamate excitotoxicity. Neuropharmacology, 53, 891–899 (2007).
5) Chen T, Fei F, Jiang XF, Zhang L, Qu Y, Hau K, Fei Z. Down-regulation of Homer1b/c attenuates glutamate-mediated excitotoxicity through endoplasmic reticulum and mitochondria pathways in rat cortical neurons. Free Radic. Biol. Med., 52, 208–217 (2012).
6) Dingledeine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol. Rev., 51, 7–61 (1999).
7) Arundine M, Tymianski M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cell. Mol. Life Sci., 61, 657–668 (2004).
8) Sun ZW, Zhang L, Zhu SJ, Chen WC, Mei B. Excitotoxicity effects of glutamate on human neuroblastoma SH-SY5Y cells via oxidative damage. Neurosci. Bull., 26, 3–16 (2010).
9) Ziegler D, Beljanovic M, Mehrnet H, Gries FA. Alpha-lipoic acid in the treatment of diabetic polyneuropathy in Germany. current evidence from clinical trials. Exp. Clin. Endocrinol. Diabetes, 107, 421–430 (1999).
10) Wollin SD, Jones PJ. Alpha-lipoic acid and cardiovascular disease. J. Nutr., 133, 3327–3330 (2003).
11) Astiz M, de Alaniz MJ, Marra CA. The oxidative damage and inflammation caused by pesticides are reverted by lipoic acid in rat brain. Neurosci. Int., 61, 1231–1242 (2012).
12) Yang TY, Xu ZF, Liu W, Xu B, Deng Y, Li YH, Feng S. Alpha-lipoic acid protects against methylmercury-induced neurotoxic effects via inhibition of oxidative stress in rat cerebral cortex. Environ. Toxicol. Pharmacol., 39, 157–166 (2015).
13) Moreira PI, Harris PL, Zhu X, Santos MS, Oliveira CR, Smith MA, Perry G. Lipoic acid and N-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer’s disease patient fibroblasts. J. Alzheimer’s Dis., 12, 195–206 (2007).
14) Melli G, Taiana M, Camozzi F, Triolo D, Podini P, Quattrini A, Taroni F, Lauria G. Al pha-lipoic acid prevents mitochondrial damage and neurotoxicity in experimental chemotheraphy neuropathy. Neurol. Sci., 214, 276–284 (2008).
15) Lee SJ, Kim SH, Kang JG, Kim CS, Ihn SH, Choi MG, Yoo HJ. Alpha-lipoic acid inhibits endoplasmic reticulum stress-induced cell death through PI3K/Akt signaling pathway in FRTL5 thyroid cells. Horm. Metab. Res., 43, 445–451 (2011).
16) Min AK, Kim MK, Kim HS, Seo HY, Lee KU, Kim JG, Park KG, Lee IK. Alpha-lipoic acid attenuates methionine choline deficient diet-induced steatohepatitis in C57BL/6 mice. Life Sci., 90, 200–205 (2012).
17) Hu H, Wang C, Jin Y, Meng Q, Liu Q, Liu K, Sun H. Alpha-lipoic acid defends homocysteine-induced endoplasmic reticulum and oxidative stress in HAEcS. Biomed. Pharmacother., 60, 63–72 (2016).
18) Chen CJ, Liao SL. Glutoxic action of glutamate on cultured astrocytes. J. Neurochem., 75, 1557–1565 (2000).
19) Han D, Sen CK, Roy S, Kobayashi MS, Tritschler HJ, Packer L. Protection against glutamate-induced cytotoxicity in C6 glial cells by thiol antioxidants. Am. J. Physiol., 273, R1771–R1778 (1997).
20) Kabadere S, Oztopcu P, Korkmaz S, Erol K, Uyar R. MgsSO4 and lazaro t(8-U-8383E6) partially protects glioma cells against glutamate toxicity in vitro. Acta Neurol. Biol., 64, 461–466 (2004).
21) Kleinkauf-Rocha J, Bobermin LD, Machado Pde M, Goncalves CA, Gottfried C, Quincozes-Santos A. Lipoic acid increases glutamate uptake, glutamine synthetase activity and glutathione content in C6 astrocyte cell line. Int. J. Dev. Neurosci., 31, 165–170 (2013).
22) Santos CL, Bobermin LD, Souza DG, Beller L, Beliver L, Arus BA, Souza DO, Goncalves CA, Quincozes-Santos A. Lipoic acid and N-acetylcysteine prevent ammonia-induced inflammatory response in C6 astroglial cells: the putative role of ERK and HO-1, signaling pathways. Toxicol. In Vitro, 29, 1350–1357 (2015).
23) Kamencic H, Lyon A, Paterson PG, Juurlink BH. Monochlorobori- manfluorometric method to measure tissue glutathione. Anal. Biochem., 286, 35–37 (2000).
24) Ren LQ, Garrett DK, Syapin M, Syapin PJ, Differential fibronectin expression in activated C6 glial cells treated with ethanol. Mol. Pharmacol., 58, 1303–1309 (2000).
25) McElnea EM, Quill B, Docherty NG, Iarnmet N, Sear S, Track AF, O’Brien CJ, Wallace DM. Oxidative stress, mitochondrial dysfunction and calcium overload in human lamina cribrosa cells from glaucoma donors. Mol. Vis., 17, 1182–1191 (2011).
26) Cheng J, Wang F, Yu DF, Wu PF, Chen JG. The cytotoxic mecha- nism of malondialdehyde and protective effect of carnosine via protein cross-linking/mitochondrial dysfunction/reactive oxygen species/MAPK pathway in neurons. Eur. J. Pharmacol., 650, 184–194 (2011).
27) Sheng WS, Hu S, Feng A, Rock RB. Reactive oxygen species from human astrocytes induced functional impairment and oxidative damage. Neurochem. Res., 38, 2148–2159 (2013).
28) Shay KP, Moreau RF, Smith EJ, Smith AK, Hagen TM. Alpha- lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. Biochim. Biophys. Acta, 1790, 1149–1160 (2009).
29) Hassain A, Ahmed AA, Shaoun SA, Sharawy S. Ameliorating effect of dl-alpha-lipoic acid against cisplatin-induced nephrotoxicity and cardiotoxicity in experimental animals. Drug Discov. Ther., 6, 147–156 (2012).
30) Mattson MP. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann. N. Y. Acad. Sci., 1144, 97–112 (2008).
31) Schneider GH, Baethmann A, Kempski O. Mechanisms of glial swelling induced by glutamate. Can. J. Physiol. Pharmacol., 70 (Suppl.), S343–S348 (1992).
32) McGeer PL, McGeer EG. Glial reactions in Parkinson’s disease. Mov. Disord., 23, 474–483 (2008).
33) Goldsmith PC. Neuroglial responses to elevated glutamate in the medial basal hypothalamus of the infant mouse. J. Nutr., 130 (Suppl.), S1032–S1038 (2000).
34) Shih AT, Erb H, Sun X, Toda S, Kalivas PW, Murphy TH. Cystine/ glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J. Neurosci., 26, 10514–10523 (2006).
35) Xu X, Chua CC, Song J, Kostrzewa RM, Kumaraguru U, Handy
RC, Chua BH. Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in HT-22 cells. *J. Neurochem.*, **103**, 2004–2014 (2007).

36) Wulfner U, Seyfried J, Groscurth P, Beinroth S, Winter S, Gleichmann M, Heneka M, Loschmann P, Schulz JB, Weller M, Klockgether T. Glutathione depletion and neuronal cell death: the role of reactive oxygen intermediates and mitochondrial function. *Brain Res.*, **826**, 53–62 (1999).

37) Merad-Boudia M, Nicole A, Santiard-Baron D, Saille C, Ceballos-Picot I. Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson’s disease. *Biochem. Pharmacol.*, **56**, 645–655 (1998).

38) Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Bachrache EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Naaz G, Peter ME, Tschopp J, Yuan J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. *Nature*, **403**, 98–103 (2000).

39) Roussel BD, Kruppa AJ, Miranda E, Crowther DC, Lomas DA, Marciniak SJ. Endoplasmic reticulum dysfunction in neurological disease. *Lancet Neurol.*, **12**, 105–118 (2013).

40) Matus S, Glimcher LH, Hetz C. Protein folding stress in neurodegenerative diseases: a glimpse into the ER. *Curr. Opin. Cell Biol.*, **23**, 239–252 (2011).

41) Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell.*, **6**, 1099–1108 (2000).

42) Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, **415**, 92–96 (2002).

43) Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. *Nature*, **403**, 98–103 (2000).

44) Kalai M, Lamkanfi M, Denecker G, Boogmans M, Lippens S, Meeus A, Declercq W, Vandenabeele P. Regulation of the expression and processing of caspase-12. *J. Cell Biol.*, **162**, 457–467 (2003).

45) Hayashi T, Rizzuto R, Hajnoczy G, Su TP. MAM: more than just a housekeeper. Regulation of the expression and processing of caspase-12. *Trends Cell Biol.*, **19**, 81–88 (2009).

46) Silva JM, Wong A, Carelli V, Cortopassi GA. Inhibition of mitochondrial function induces an integrated stress response in oligodendroglia. *Neurobiol. Dis.*, **34**, 357–365 (2009).

47) Appenzeller-Herzog C. Glutathione and non-glutathione-based oxidant control in the endoplasmic reticulum. *J. Cell Sci.*, **124**, 847–855 (2011).

48) Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr. Opin. Cell Biol.*, **19**, 142–149 (2007).

49) Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M. Astrocytes and stroke: networking for survival? *Neurochem. Res.*, **28**, 293–305 (2003).

50) Ricer G, Volpi L, Pasquali L, Petrozzi L, Siciliano C. Astrocyte-neuron interactions in neurological disorders. *J. Biol. Phys.*, **35**, 317–336 (2009).

51) Fawcett JW, Asher RA. The glial scar and central nervous system repair. *Brain Res. Bull.*, **49**, 377–391 (1999).

52) Rocamonde B, Paradells S, Barcia C, Garcia Esparza A, Soria JM. Lipoic acid treatment after brain injury: study of the glial reaction. *Clin. Dev. Immunol.*, **2013**, 521939 (2013).

53) Zhang YH, Wang DW, Xu SF, Zhang S, Fan YG, Yang YY, Guo SQ, Wang S, Guo T, Wang ZY, Guo C. α-Lipoic acid improves abnormal behavior by mitigation of oxidative stress, inflammation, ferroptosis, and tauopathy in P301S Tau transgenic mice. *Redox Biol.*, **14**, 535–548 (2018).