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Effects and mechanism of lipoic acid on beta-amyloid-intoxicated C6 glioma cells

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ABSTRACT. β-amyloid peptides (Aβs) can exert neurotoxic effects through induction of oxidative damage, whereas lipoic acid (LA), a powerful antioxidant, can alleviate oxidative damage. In this study, we explored the effect and mechanism of action of LA on beta-amyloid-intoxicated C6 glioma cells. Cells were randomly divided into three groups: control (vehicle), Aβ, and LA + Aβ. The LA + Aβ group was treated with LA for 2 h, then both the Aβ-only and the LA + Aβ groups were incubated with 25 μM Aβ for 24 h. Cell viability was measured by the MTT method. Mitochondrial reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were detected by enzyme-linked immunosorbent assay (ELISA), and the GSH to GSSG ratio calculated. Real-time polymerase chain reaction and western blot analyses were used to detect MnSOD mRNA and protein, respectively. Aβ

significantly inhibited C6 cell proliferation compared with the control group ($P < 0.05$). LA markedly increased cell viability compared with the Aβ group ($P < 0.05$). The increased GSSH and decreased GSH mitochondrial accumulation induced by Aβ was profoundly reversed by treatment with LA ($P < 0.05$). Aβ significantly reduced *MnSOD* expression compared to controls ($P < 0.05$), whereas LA pretreatment increased *MnSOD* expression compared with the Aβ-only group ($P < 0.05$); MnSOD protein levels showed similar patterns. These results suggest that LA might protect Aβ-intoxicated C6 glioma cells by alleviating oxidative damage, providing a new treatment strategy for neurodegenerative diseases.

Key words: Lipoic acid; β-amyloid; C6 glioma cells; Oxidative stress

INTRODUCTION

The improvement of living conditions and the prolonged average life expectancy have gradually resulted in an ageing society. Concomitantly, the incidence of neurodegenerative diseases such as Alzheimer's disease (AD) has increased significantly (Choi et al., 2014a). As one of the common disorders of the central nervous system seen in the elderly, AD leads to a progressive degeneration of memory, cognition, and reasoning, which seriously affects the quality of life of the elderly and endangers their health. AD presents a difficult medical problem across many clinical disciplines including neurology and gerontology (Willhite et al., 2014). Its pathological changes primarily consist of brain tissue atrophy with a large amount of β-amyloid peptide (Aβ) deposition in the brain tissue and brain blood vessels, and the presence of senile plaques and neurofibrillary tangles in hippocampal neurons (Pereira et al., 2014) Studies have shown that multiple factors including the destruction of calcium homeostasis, apolipoprotein E, free radical damage, metabolic disorders, and cholinergic injury might induce AD (Arevalo-Rodriguez et al., 2014; Bazin and Bratu, 2014; Savioz et al., 2014). However, the pathogenesis of AD has not been fully elucidated.

Aβ, the hydrophobic peptide consisting of 39-43 amino acids cleaved from an amyloid precursor, can be found in normal cerebrospinal fluid and in AD tissue as an inherent soluble component of cells. Neurotoxic accumulations of Aβ form the core of senile plaques; therefore, Aβ deposition is considered to be one of the main pathological mechanisms of AD (Cho et al., 2014). Pathological deposition of Aβ can cause dementia, and can activate neuronal apoptosis and damage mitochondria by releasing and accumulating reactive oxygen species (ROS) (Forny-Germano et al., 2014; Jiang et al., 2014). ROS are primarily produced by mitochondria and can cause oxidative stress with subsequent neuronal death, demonstrating that ROS is also a key factor of AD (Yang and Cui, 2014).

Lipoic acid (LA) is characterized as a strong water and fat soluble antioxidant, and is known as a human "natural antioxidant". LA, a member of the B vitamin family, is a coenzyme in the multienzyme complexes of dehydrogenase and aminocaproic acid decarboxylase (Asci et al., 2014). Recent studies have shown that LA can play an important protective role in diabetes, diabetic complications, and in ischemia reperfusion injury of the heart, brain, and kidney by removing toxic chemicals, scavenging free radicals and ROS, and improving blood

circulation (Bingham et al., 2014; Tanaka et al., 2014; Zhang et al., 2014). This study was designed to investigate the protective effect and mechanism of LA on C6 cell injury induced by Aβ.

MATERIAL AND METHODS

Materials

C6 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Lipoic acid and Aβ1-42 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aβ1-42 was dissolved in deionized distilled water at a concentration of 500 μM and kept at -20°C until use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin (100 U/mL)/streptomycin (100 U/mL) and ethylenediaminetetraacetic acid (EDTA) were purchased from Hyclone (Logan, UT, USA). Dimethyl sulfoxide and MTT powder were purchased from Gibco (Gaithersburg, MD, USA). Trypsin-EDTA solution was purchased from Sigma-Aldrich. The mitochondrial protein extraction kit was purchased from Wuhan Boster Biotechnology Co. Ltd. (Wuhan, China). Glutathione (GSH) and oxidized glutathione (GSSG) ELISA kits and western blot reagents were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Polyvinylidene fluoride (PVDF) membranes were purchased from Pall Life Sciences (Port Washington, NY, USA). TRIzol, the RNA extraction kit, and real-time polymerase chain reaction (PCR) reagents were obtained from Invitrogen (Carlsbad, CA, USA). ECL was purchased from Amersham Biosciences (Piscataway, NJ, USA). The rabbit anti-human MnSOD monoclonal antibody and mouse anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Other reagents were purchased from Shanghai Sangon Biotechnology Co., Ltd. The Labsystem Version 1.3.1 enzyme was purchased from Bio-Rad Laboratories (Berkeley, CA, USA). The clean bench was purchased from Suzhou Tsu Purification Equipment Engineering Co., Ltd. (Suzhou, China).

Cell culture and subgrouping

C6 cells were cultured in DMEM supplemented with FBS (10%) and penicillin (100 U/mL)/streptomycin (100 U/mL) with 5% CO_2 and 95% O_2 at 37°C. Prior to the initiation of treatments, 1 x 10^6 C6 cells/cm^2 were seeded on culture dishes. C6 glioma cells were randomly divided into three groups: control (vehicle), Aβ only, and LA + Aβ. C6 cells in the Aβ group were treated with 25 μM Aβ for 24 h, and C6 cells in the LA + Aβ group were first incubated with 50 μM LA for 2 h followed by incubation with 25 μM Aβ for 24 h (Feng and Zhang, 2004; Di Domenico et al., 2014).

MTT assessment of cell proliferation

Cells in each group were digested and counted, then cultured on 96-well plates at 3000 cells/well. Subsequently, 20 μL MTT (5 g/L) was added to each well and the cells cultured for 4 h in an incubator. DMSO (150 μL/well) was added after discarding the culture medium, and then the plates were shaken for 10 min until the purple crystals were fully dissolved. Absor-

bance was detected by a microplate reader (Thermo Scientific) at 492 nm to calculate the cell proliferation rate (%). The experiment was repeated three times.

Isolation of mitochondria from C6 cells

A trypsin-EDTA solution was used to dissociate the C6 cells, which were harvested with phosphate buffered saline (PBS) by centrifugation at 800 *g* for 5 min. An aliquot of 5 x 10^7 cells was suspended in 2.5 mL pre-cooled mitochondrial isolation buffer and incubated for 10 min at room temperature. The cell membranes were ruptured by ultrasound. The cell homogenate was centrifuged at 800 *g* for 5 min at 4°C, and then centrifuged at 12,000 *g* for 10 min. After removal of the supernatant, the pellet (containing the mitochondria) was collected. The isolated mitochondria were washed again using 0.5 mL mitochondrial isolation buffer.

Detection of GSH and GSSG in the mitochondria of C6 cells

An ELISA kit was used to determine the levels of GSH and GSSG in the mitochondria of C6 cells. The procedures were performed according to the instructions for the GSH and GSSG assay kits. Briefly, the cells were washed with PBS, trypsinized, and centrifuged for collection. Then, the pellets were suspended in protein removal reagent M, lysed by freeze-thawing twice, and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was collected for measurement of total GSH. Supernatant aliquots (10 µL) were incubated at room temperature for 5 min with the addition of 150 µL total GSH test solution. The sample absorbance was measured at 412 nm after the addition of 50 µL NADPH. After adding a GSH removal agent, the GSSG was measured and the ratio of GSH and GSSG was calculated.

Detection of *MnSOD* mRNA expression in the mitochondria of C6 cells by real-time PCR

Total RNA in the mitochondria of 2.5 x 10^7 C6 cells in each group was isolated and reverse-transcribed to generate cDNA. The primer sequences used for real-time PCR were as follows: *MnSOD* forward primer: 5'-TGA CCT GCC TTA CGA CTA TGG-3'; *MnSOD* reverse primer: 5'-AAC TCT CCT TTG GTT CTC CA-3'; *GAPDH* forward primer: 5'-AGT ACC AGT CTG TTG CTG G-3'; *GAPDH* reverse primer: 5'-TAA TAG ACC CGG ATG TCT GGT-3'. The cDNA was used as the template for PCR on an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a total volume of 20 µL containing 0.5 mm of each primer, 10 µL SYBRw Green and 0.5 mm of cDNA. The PCR conditions were as follows: 50°C for 1 min; 95°C for 30 s; followed by 35 cycles of 56°C for 50 s; and 72°C for 35 s. The initial cycle threshold numbers (Cts) of all standards and samples were calculated by the fluorescent quantitative PCR software (REST, Michael, Munich, Germany) with *GAPDH* as the reference. Quantitative analysis of samples was performed using a standard curve according to the standard Ct and $2^{-\Delta Ct}$ calculation.

Detection of MnSOD protein expression in the mitochondria of C6 cells by western blot

Each mitochondrial sediment sample was lysed for 40 min at 4°C by vigorous shaking in RIPA buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 150 mM NaCl, 1 mM dithiothreitol, 50 mM Tris-HCl, pH 7.4, and protease inhibitors), and then centrifuged at 12,000 *g* for 10 min. The supernatant was collected and the protein concentrations therein were detected. Each protein sample (25 μg) was analyzed with 10% SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked using fresh 5% nonfat dry milk dissolved in Tris-buffered saline (TBS). The MnSOD antibody at 1:500 dilution was added to the membrane and incubated at 4°C overnight then washed with PBS plus Tween 20 (PBST) three times, followed by addition of 1:2000 secondary antibody with a second incubation at room temperature for 1 h. After washing three times with TBS plus Tween 20 (TBST), the membrane was incubated with enhanced ECL for 1 min and exposed using X-ray film. The X-ray film was scanned using a Bio-Rad system (Molecular Imager GS-800™ calibrated densitometer). The band density was measured by the Quantity one software (Bio-Rad) with actin as an internal control. Experiments were repeated four times (N = 4).

Statistical analysis

The SPSS software 16.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. All data are reported as means ± SD. Differences between groups were determined using one-way analysis of variance (ANOVA) and chi-square tests. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of LA on C6 glioma cell proliferation

The results of our analyses showed that Aβ clearly inhibited C6 cell proliferation, and that the difference between the Aβ and control groups was statistically significant ($P < 0.05$). Compared with the Aβ-only group, LA promoted C6 cell viability after their damage by Aβ ($P < 0.05$). Notably, the viability did not differ significantly between the LA + Aβ and the control groups ($P > 0.05$; Figure 1), which indicates that LA can protect Aβ-intoxicated C6 glioma cells and promote the recovery of viability.

GSH and GSSG changes in the mitochondria of C6 cells

In this study, Aβ peptides were shown to cause GSH decreases and GSSG increases, and could further significantly decrease the ratio of GSH to GSSG compared to the control group ($P < 0.05$). However, after LA treatment, the ratio of GSH to GSSG was significantly increased as compared to the Aβ group ($P < 0.05$; Table 1), which suggested that Aβ peptides can damage C6 cells through oxidative stress, whereas LA can reverse the Aβ effect through alleviation of the oxidative stress-induced damage.

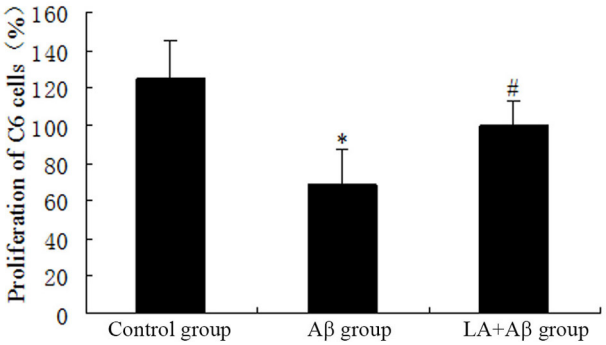

Figure 1. Effect of LA on the proliferation of C6 glioma cells. *Compared with the control group, $P < 0.05$; #compared with the Aβ group, $P < 0.05$. Aβ = β amyloid peptide; LA = lipoic acid. Error bars represent standard deviation.

Table 1. Changes of GSH and GSSG in the mitochondria of C6 cells.

Group	GSH (μM)	GSSG (μM)	GSH/GSSG
Control	5.43 ± 0.98	2.78 ± 0.49	2.11 ± 0.53
Aβ	2.25 ± 0.62*	4.43 ± 0.33*	0.95 ± 0.15*
LA + Aβ	5.12 ± 0.79#	2.92 ± 0.27#	1.87 ± 0.28#

*Compared with the control group, $P < 0.05$; #compared with the Aβ group, $P < 0.05$. GSH = glutathione; GSSG = oxidized glutathione; Aβ = β amyloid peptide; LA = lipoic acid. Values are reported as means ± standard deviation.

Changes of *MnSOD* mRNA in the mitochondria of C6 cells

The results showed that *MnSOD* mRNA expression in the mitochondria of C6 cells markedly decreased after the cells were exposed to Aβ; this difference was statistically significant ($P < 0.05$). In contrast, LA pretreatment could significantly increase the expression of *MnSOD* mRNA compared to that in the Aβ-only group ($P < 0.05$; Figure 2).

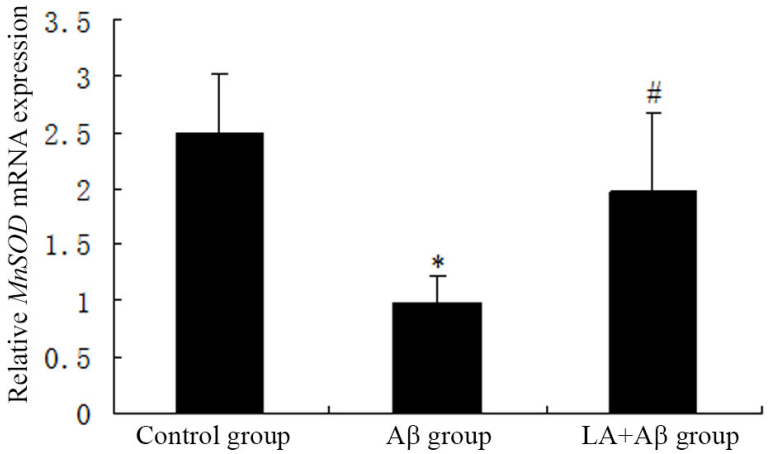

Figure 2. Changes of *MnSOD* mRNA in the mitochondria of C6 cells. *Compared with the control group, $P < 0.05$; #compared with the Aβ group, $P < 0.05$. MnSOD = mitochondrial antioxidant manganese superoxide dismutase; Aβ = β amyloid peptide; LA = lipoic acid.

Changes of the MnSOD protein in the mitochondria of C6 cells

The results showed that the levels of the MnSOD protein in the mitochondria of C6 cells significantly decreased after exposure to Aβ ($P < 0.05$). On the other hand, LA pretreatment led to significantly increased expression of the MnSOD protein over that of the Aβ-only group ($P < 0.05$; Figure 3). These results suggested that MnSOD protein changes were consistent with those of *MnSOD* mRNA that occurred during the injury process by Aβ and consequent to LA pretreatment.

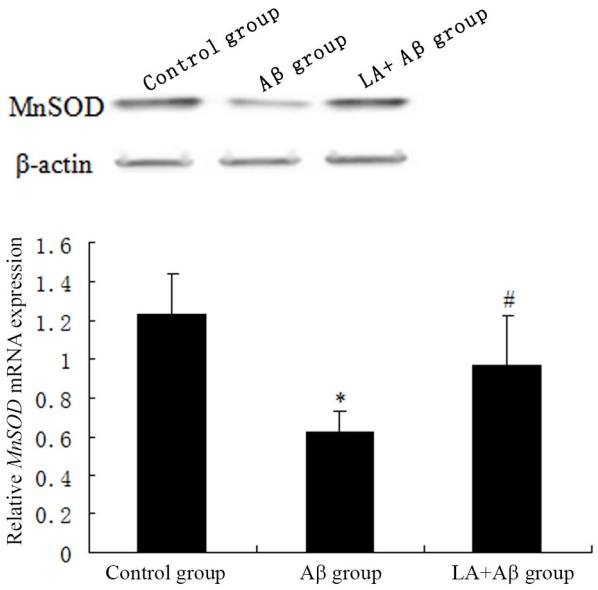

Figure 3. Changes of the MnSOD protein in the mitochondria of C6 cells. *Compared with the control group, $P < 0.05$; #compared with the Aβ group, $P < 0.05$. MnSOD = mitochondrial antioxidant manganese superoxide dismutase; Aβ = β amyloid peptide; LA = lipoic acid.

DISCUSSION

Several studies have confirmed that Aβ1-42 is highly hydrophobic, readily accumulates in cells, and forms precipitation inclusion bodies and fibrillary tangles, inducing cell toxicity. The dynamic balance of the production and removal of Aβ in normal brain tissues is maintained by the blood-brain barrier transport mechanism and by degradation within the brain. However, the formation of lesions in brain tissue results in disruption of this balance. Therefore, cells become overloaded with Aβ that is generated but cannot be cleared. The majority of this excess Aβ is deposited to form neurotoxic senile plaques, considered to be one of the major causes of AD (Eleuteri et al., 2014; Mhatre et al., 2014). Thus, Aβ generation and accumulation is a key feature of AD pathogenesis.

Many studies have shown that mitochondrial function damage and oxidative stress can lead to the occurrence of neurodegenerative diseases such as AD. Aβ may increase ROS production and the concentration of free radicals, resulting in lipid peroxidation and increased

cell membrane permeability. Oxidative stress occurs earlier than does the formation of senile plaques in AD, suggesting that oxidant stress plays a role in Aβ generation as well as in its accumulation. Mitochondrial membrane rupture caused by oxidative stress allows calcium to flow into the cytoplasm, activating calcium dependent proteases and increasing intracellular free radicals. Such further damage to mitochondrial function is expected to result in neuronal energy metabolism disorders and aggravation of neurological degenerative diseases (Choi et al., 2014b; Hanenberg et al., 2014). LA, a powerful antioxidant, can inhibit the generation of oxygen free radicals and thus can regulate GSH. This suggested that LA might have certain protective effects against diseases induced by oxygen free radicals (Bhadri et al., 2013; Guo et al., 2014; Patel et al., 2014). Our study demonstrated that Aβ peptides could significantly inhibit the proliferation of C6 cells, while LA could reverse the inhibition effect of Aβ and promote the recovery of cell viability. Aβ exposure was also found to decrease the ratio of GSH and GSSH, and it reduced the expression of both MnSOD mRNA and protein in the mitochondria of C6 cells. However, LA pretreatment of Aβ-intoxicated C6 glioma cells could reverse the expression changes seen in GSH and GSSH, increase the ratio of GSH and GSSH, and increase the expression levels of MnSOD mRNA and protein, restoring these to approximately control levels.

In summary, the results of this study suggested that Aβ exposure injured C6 cells through oxidative stress and oxidative damage in the mitochondria, which effect could be reversed by LA treatment, thereby further suggesting that LA might protect Aβ-intoxicated C6 cells by alleviating oxidative damage. Our results confirmed the neuroprotective effect of LA and provided evidence of its mechanism; LA thus might provide a new treatment choice for neurological degenerative diseases.

Conflicts of interest

The authors declare no conflict of interest.

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