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Isoliquiritigenin isolated from *Glycyrrhiza uralensis* protects neuronal cells against glutamate-induced mitochondrial dysfunction

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ABSTRACT

Glutamate-mediated excitotoxicity, which is associated with reactive oxygen species (ROS), is hypothesized to be a major contributor to pathological cell death in the mammalian central nervous system, and to be involved in many acute and chronic brain diseases.

Here, we showed that isoliquiritigenin (ISL) isolated from *Glycyrrhiza uralensis* (Gu), one of the most frequently prescribed oriental herbal medicines, protected HT22 hippocampal neuronal cells from glutamate-induced oxidative stress. In addition, we clarified the molecular mechanisms by which it protects against glutamate-induced neuronal cell death. ISL reversed glutamate-induced ROS production and mitochondrial depolarization, as well as glutamate-induced changes in expression of the apoptotic regulators Bcl-2 and Bax. Pretreatment of HT22 cells with ISL suppresses the release of apoptosis-inducing factor from mitochondria into the cytosol. Taken together, our results suggest that ISL may protect against mitochondrial dysfunction by limiting glutamate-induced oxidative stress.

In conclusion, our results demonstrated that ISL isolated from Gu has protective effects against glutamate-induced mitochondrial damage and hippocampal neuronal cell death. We expect ISL to be useful in the development of drugs to prevent or treat neurodegenerative diseases.

1. Introduction

Glutamate is an endogenous excitatory neurotransmitter in the mammalian central nervous system. Glutamate-mediated excitotoxicity, which is associated with reactive oxygen species (ROS), may be a major contributor to pathological cell death within the nervous system and may be involved in many acute and chronic brain diseases [1,2].

HT22 cell, an immortalized mouse hippocampal neuron cell line, has been widely used in various *in vitro* models to study the mechanism of glutamate-induced neurotoxicity. The results of various studies have suggested that glutamate induces oxidative stress in HT22 cells [3–5]. Notably, increased ROS levels mediate glutamate-induced cell death of HT22 cells because these cells do not express functional ionotropic receptors, such as the *N*-methyl-D-aspartate (NMDA) receptor [6]. The main mechanisms of glutamate-induced neuronal cell death include increased intracellular ROS generation, loss of mitochondrial membrane potential ($\Delta\psi_m$), and release and translocation of AIF from mitochondria into the cytosol as a result of abnormal levels of mitochondrial proteins, such as Bcl-2 and Bax, which regulate apoptotic programmed cell death. Glutamate-induced apoptosis in HT22 cells is facilitated by the altered expression and mitochondrial localization of apoptotic regulators, e.g., down-modulation of the antiapoptotic protein Bcl-2 and upregulation of the proapoptotic protein Bax [7]. Therefore, HT22 cells provide a good model system in which to study neuronal cell degeneration as well as screening for agents that may prevent glutamate-induced cell death, such as antioxidants [8].

Glycyrrhizae radix (GR, licorice) is comprised of the dried roots of *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and other *Glycyrrhiza* species [9]. Among these, *G. glabra* (Gg) and *G. uralensis* (Gu) are the most commonly used plants. GR is one

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of the most frequently prescribed oriental herbal medicines, and has been used for thousands of years for various purposes, such as detoxification, moistening of the lungs, cough relief, reducing inflammation, and in the treatment of gastric ulcers [10,11]. Several studies have investigated the constituents of GR, which include saponins, glycyrrhizin, glycyrrhetinic acid, liquiritin, liquiritigenin, isoliquiritin, and isoliquiritigenin (ISL). The biological activities of compounds isolated from GR have also been reported. Flavonoids have antitussive, antiallergic, anticancer, and antiinflammatory activities [12–14]. Glabridin, the major isoflavone in Gg, which is mainly consumed in Europe and Russia, exhibits potent effects on cognitive impairment in diabetes and middle cerebral artery occlusion (MCAo) rats [15,16]. Neuroprotective effects of Gu, which serves as another major plant source of GR in the Far East, have also been reported. For example, the traditional Oriental medicine Insampaedok-san and its fermented extract, which contains Gu, showed protective effects against glutamate-induced neurotoxicity in HT22 cells [17]. Although Gu does not contain glabridin [18], it may contain other neuroprotective compounds. This prompted us to search for neuroprotective compounds in Gu.

Here, we isolated an active compound from Gu, which we identified as ISL by HPLC and ^{1}H and ^{13}C nuclear magnetic resonance (NMR). We then examined whether ISL protects HT22 hippocampal neuronal cells from glutamate-induced oxidative stress. We subsequently clarified the molecular mechanisms by which it protects against glutamate-induced neuronal cell death.

2. Materials and methods

2.1. Isolation of isoliquiritigenin (ISL) from G. uralensis (Gu)

G. uralensis (Gu) was purchased from Daeyu Oriental Pharm Co. (Daegu, Korea). The specimen was stored at the Natural Products Chemistry Laboratory, Kyungpook National University, Daegu, Korea (voucher specimen number; KNUNPC-GR-06-001). Analytical grade of ethanol (EtOH), dichloromethane (CH_2Cl_2), *n*-hexane, ethylacetate (EtOAc), and acetone were used for extraction and column chromatography (Duksan Chemical, Korea). For structure determination, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Digital 400 NMR spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Proton chemical shifts were referenced using tetramethylsilane (TMS) as an internal reference, and carbon chemical shifts were referenced to the solvent. Chemical shifts (δ) were expressed in ppm relative to the TMS. Thin layer chromatography was performed on a pre-coated silica gel plate (Kieselgel 60F254, Merck, NJ) and silica gel column chromatography was carried out using Kieselgel 60 (Merck, NJ).

2.2. HT22 cell culture

HT22 cells, a mouse hippocampus-derived cell line, were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml of penicillin streptomycin (WelGENE, Korea) at 37 °C in 5% CO_2.

2.3. MTT reduction assay

HT22 cells (1.0×10^4) were seeded in 96-well plates. Following 24 h of incubation, they were treated with or without ISL for 12 h, and then treated with 10 mM glutamate for 12 h. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The absorbance was read with a microplate reader (TECAN, Austria) at 570 nm. The percentage of surviving cells was measured relative to the control values.

2.4. Flow cytometry

2.4.1. FITC-annexin V and propidium iodide double staining

HT22 cells (2×10^5) were seeded into 6-well plates. Following 24 h of incubation, they were treated with or without ISL for 12 h, and then treated with 10 mM glutamate. Twelve hours after glutamate treatment, the cell pellet was prepared in annexin V-FITC solution and incubated in 5% CO_2 at 37 °C. The PI solution was then added, and the ratio of PI-positive and annexin V-positive cells was determined by flow cytometry (FACSCalibur; BD Biosciences, NJ).

2.4.2. Analysis of intracellular ROS

Intracellular ROS were detected by 2,7-dichlorofluorescein diacetate (CM-H_2DCF-DA) (Invitrogen, CA). Twenty four hours after glutamate treatment, the HT22 cells were loaded with 10 μM CM-H_2DCF-DA for 30 min and the fluorescence at 530 nm was monitored by flow cytometry (FACSCalibur; BD Biosciences, NJ) at an excitation wavelength of 488 nm.

2.4.3. Analysis of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondria membrane potential ($\Delta\psi_m$) of the HT22 neurons was determined by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Invitrogen, CA) reduction. JC-1 is a sensitive indicator of $\Delta\psi_m$, which can be turn to green from red fluorescence. HT-22 neurons were stained with JC-1 according to the manufacturer's protocol [8] and analyzed by subsequent flow cytometry (FACSCalibur; BD Biosciences, NJ). JC-1 red fluorescence indicating intact $\Delta\psi_m$ was excited at 488 nm and emission was detected using a 613 ± 20 nm band pass filter. For each sample, 10,000 cells were acquired and analyzed by flow cytometry (FACSCalibur; BD Biosciences, NJ). Data were analyzed using the fluorescence intensity of the analyzed cell population.

2.5. Isolation of mitochondria from HT22 cells

HT22 cells were incubated for 12 h in medium containing various concentrations of ISL and then exposed to 10 mM glutamate for 24 h. 2.0×10^7 cells were harvested each sample. The Mitochondria Isolation Kit for Cultured Cells (Pierce, IL) was used to isolate mitochondria from HT22 cells in accordance with the manufacturer's instructions. Mitochondria were isolated under sterile conditions at 4 °C.

2.6. Western Blotting analysis

After the cells were harvested, cell extracts were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes (Amersham, NJ). The blots were incubated with the following antibodies: anti-Bcl-2, Bax, AIF (Santa Cruz Biotechnology, CA), and anti-β-actin (AbFrontier, Korea). Thereafter, peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Thermo Scientific, MA) were used as secondary antibodies. The signal was developed using the enhanced chemiluminescence detection method (Thermo Scientific, MA).

3. Results

3.1. Isolation of ISL from Gu and structure determination

Dried Gu (250 g) was refluxed with 20 l of 95% EtOH for 3 h and the extract was filtered through filter paper (Advantec MFS, CA).

The filtrate was then dried under reduced pressure with a rotary evaporator. The ethanolic extract (41.92 g) was suspended in distilled water and successively partitioned with CH_2Cl_2. The organic solvent fraction was collected and concentrated with a rotary evaporator. The active CH_2Cl_2-soluble fraction (8.86 g) was subjected to open column chromatography with silica gel [3.8 × 50 cm, *n*-hexane:EtOAc = 30:1–1:1] to yield 5 fractions (Fr. 1–5). Compound **1** (6 mg) was isolated from Fr. 3 (1.20 g) by chromatography with a silica gel column (4 × 28 cm, *n*-hexane:acetone = 8:1–1:1).

The NMR data (*Fig. S1*) of Compound **1** (yellow powder) showed good agreement with previous NMR data for ISL [19]. Compound **1** was identified as ISL, and its structure is shown in Fig. 1A.

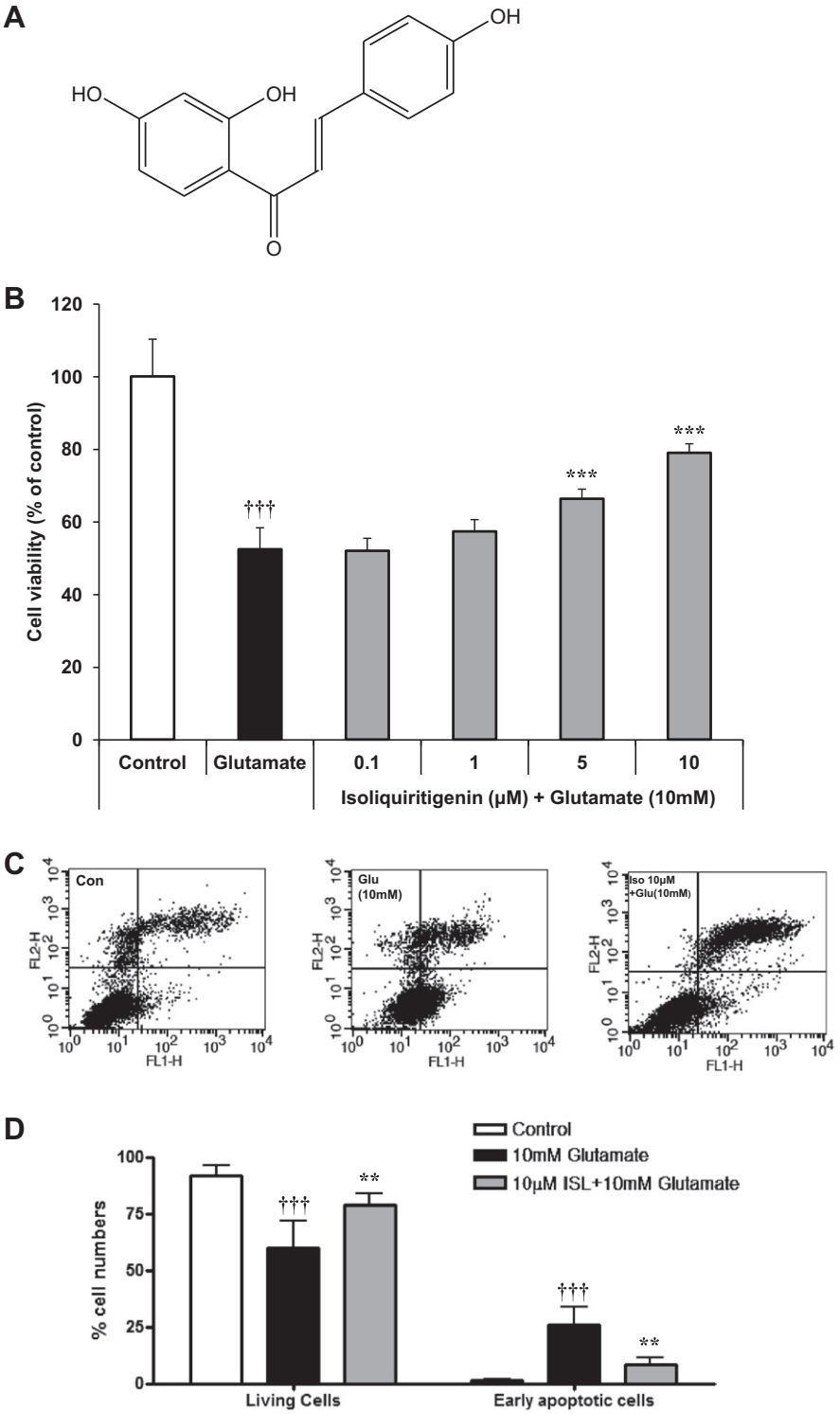

Fig. 1. ISL decreases the number of apoptotic cells due to glutamate-induced neuronal cell death. (A) Chemical structure of ISL. (B) Cell viability was assessed by the MTT reduction assay. (C) Cells were trypsinized, loaded with annexin V, and then analyzed by flow cytometry. (D) The numbers of living cells and early apoptotic cells are expressed as percentages of the total cell number. Values calculated as percentages of control cells are presented as the means ± SEM ($n = 3$). $^{\dagger\dagger\dagger}P < 0.0001$ vs. control, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. 10 mM glutamate (ANOVA, Scheffé's test).

3.2. Glutamate-induced HT22 neuronal cell death was reduced by ISL from Gu

First of all, the cytotoxicity of the isoliquiritigenin to HT22 cells was performed at 0–20 μM concentration. These results examined that isoliquiritigenin up to 10 μM for 12 h did not appear the cytotoxic effects (Fig. S2). To investigate whether ISL protects against glutamate-induced neuronal cell death, we incubated HT22 cells with or without glutamate (10 mM) and with or without ISL at concentrations ranging from 0.1 to 10 μM. ISL dose-dependently reduced glutamate-induced cell death at concentrations ranging from 1 to 10 μM (Fig. 1B). Based on these data, we used ISL at a concentration of 10 μM in subsequent experiments.

Next, we further examined the role of ISL in neuronal apoptosis, which is the main type of cell death in glutamate-induced neurotoxicity [20]. Glutamate-induced apoptosis in HT22 cells was analyzed by annexin V/propidium iodide dual staining followed by flow cytometry (Fig. 1C). As shown in Fig. 1D, treatment of HT22 cells with glutamate for 12 h increased the number of annexin V(+)/propidium iodide(−)-dual-stained (early apoptotic) cells (by about 25.8%) as compared to control cells, whereas the number of stained cells after treatment with 10 μM ISL plus glutamate was significantly decreased (by approximately 8.4%) compared to HT22 cells treated with glutamate alone. Taken together, these results indicated that 10 μM ISL effectively prevents glutamate-induced apoptosis in HT22 neuronal cells.

3.3. ISL from Gu attenuated glutamate-induced ROS production in neuronal cells

A number of studies have indicated that flavonoids with chalcone structures, such as ISL, have the capacity to act as antioxidants [21]. Therefore, we examined whether ISL suppresses glutamate-induced ROS production in HT22 cells. The intracellular ROS levels were determined in HT22 cells treated with or without 10 mM glutamate and 10 μM ISL by FACS analysis following CM-H_2DCF-DA staining. Intracellular ROS levels were increased 3.9-fold by treatment with 10 mM glutamate compared with untreated

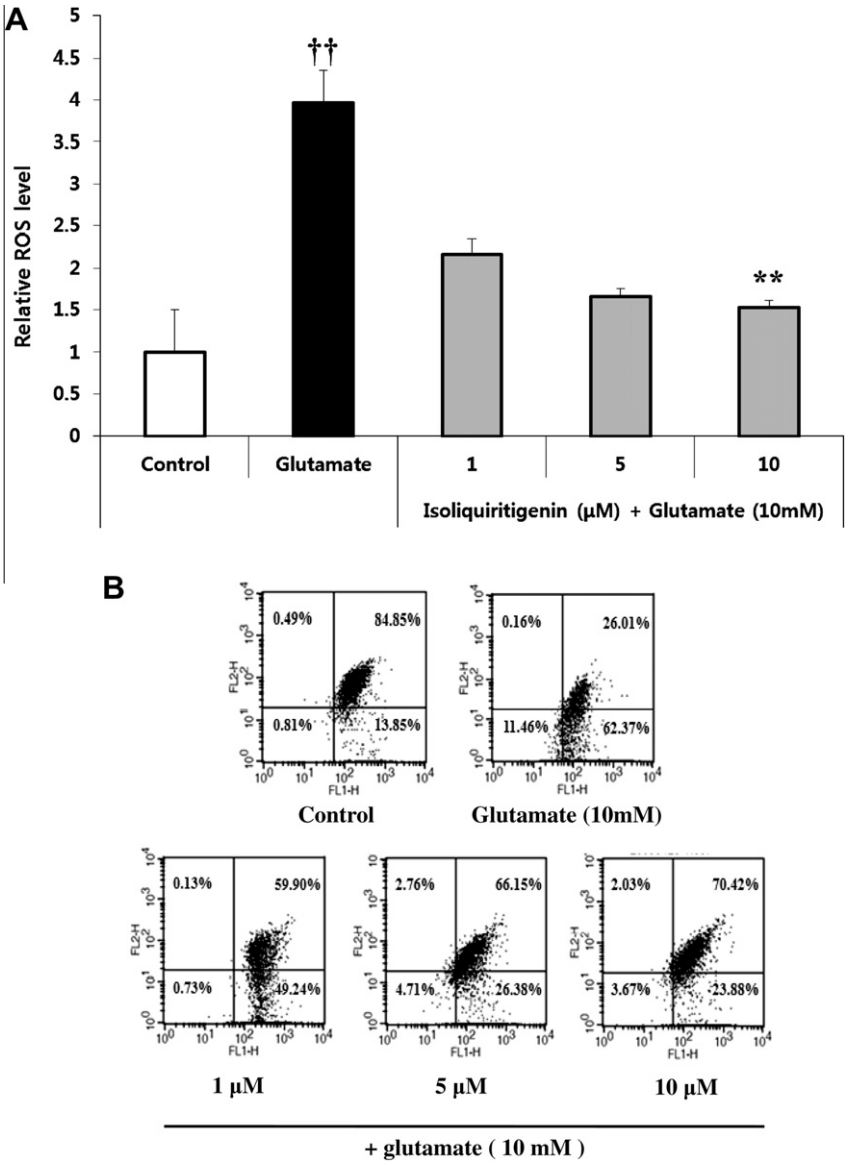

Fig. 2. ISL prevents glutamate-induced intracellular ROS formation and mitochondrial depolarization. (A) Intracellular ROS generation was significantly increased when HT22 cells were treated with 10 mM glutamate for 12 h. Levels of ROS were measured with DCF fluorescence. Values calculated as fluorescence intensity with respect to the control. Data are the means ± SEM ($n = 3$). $^{\dagger\dagger}P < 0.001$ vs. control, $^{**}P < 0.01$ vs. 10 mM glutamate (ANOVA, Scheffé's test). (B) Mitochondrial membrane potential ($\Delta\psi_m$) was analyzed by JC-1 fluorescence.

control cells. In cells treated with 10 μM ISL, glutamate treatment increased intracellular ROS levels by only 1.5-fold compared with untreated control cells (Fig. 2A). These results suggested that ISL may play a role as an intracellular ROS suppressor, and that this function may be related to the prevention of glutamate-induced apoptosis in HT22 neuronal cells.

3.4. ISL inhibited glutamate-induced mitochondrial depolarization in HT22 cells

Although the detailed mechanisms remain to be fully clarified, glutamate induces depolarization of $\Delta\psi_m$ in HT22 cells [22]. As shown in Fig. 2B, ISL protected against significant loss of red fluorescence, which reflects glutamate-induced loss of $\Delta\psi_m$. ISL prevented glutamate-induced loss of $\Delta\psi_m$, suggesting that it suppressed ROS accumulation in HT-22 cells.

3.5. Glutamate-induced changes in mitochondrial Bcl-2 and Bax levels in HT22 cells were reversed by ISL

Glutamate treatment alters the mitochondrial localization of apoptotic regulators, such as the antiapoptotic protein, Bcl-2, and the proapoptotic protein, Bax [7]. Therefore, we measured Bcl-2 and Bax protein levels in mitochondria and the cytosol to investigate whether ISL regulates the translocation of Bcl-2 and Bax. Western Blotting analysis of the mitochondrial fraction showed that glutamate mitochondrial Bcl-2 levels decreased and mitochondrial Bax levels increased (Fig. 3A). In contrast, the cytosolic Bcl-2 levels increased and cytosolic Bax levels reduced (Fig. 3B). These results indicated that glutamate modulates apoptosis through its effects on mitochondrial antiapoptotic and proapoptotic factors. These glutamate-induced changes in Bcl-2 and Bax protein levels were reversed by ISL (10 μM, Fig. 3C). In the absence of glutamate, ISL did not alter the levels of Bcl-2 or Bax (data not shown). These results suggested that ISL may limit mitochondrial dysfunction resulting from glutamate-induced oxidative stress, and may prevent oxidative stress-induced apoptosis in HT22 cells.

3.6. ISL prevents the release of AIF from mitochondria

Glutamate-induced cell death in neurons involves mitochondrial damage and release of proapoptotic mitochondrial proteins, such as apoptosis-inducing factor (AIF) [23]. As increased cytosolic AIF levels and reduced mitochondrial AIF levels are associated with

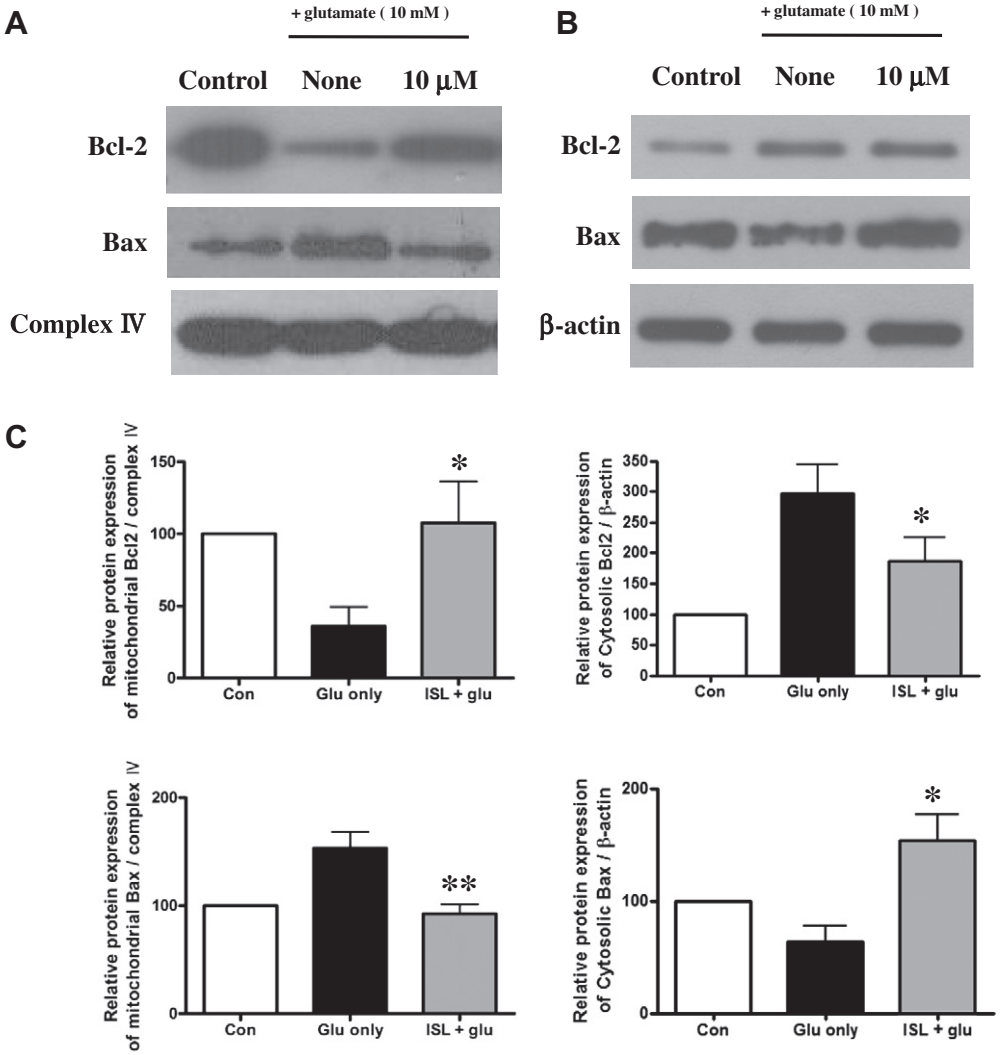

Fig. 3. ISL regulates translocation of anti- and proapoptotic regulators, Bcl-2 and Bax. Subcellular proteins were isolated from (A) mitochondria and (B) cytosol. Bcl-2 and Bax protein levels were determined by Western Blotting analysis. (C) The relative abundance of each band was quantified by intensity measurements of Bcl-2 and Bax in contrast to control of mitochondrial (ComplexIV) and cytosolic (β-actin) fraction, respectively. HT22 cells were treated with 10 mM glutamate for 24 h (Glu only), and without treated (Control), and pre-treated 10 mM ISL for 12 h before 10 mM glutamate-treatment for 24 h (ISL + glu). The adjusted mean ± SEM. (n = 3) of each band is shown above each blot. *P < 0.05 compared to 10 mM glutamate-treated cells (ANOVA, Scheffé's test).

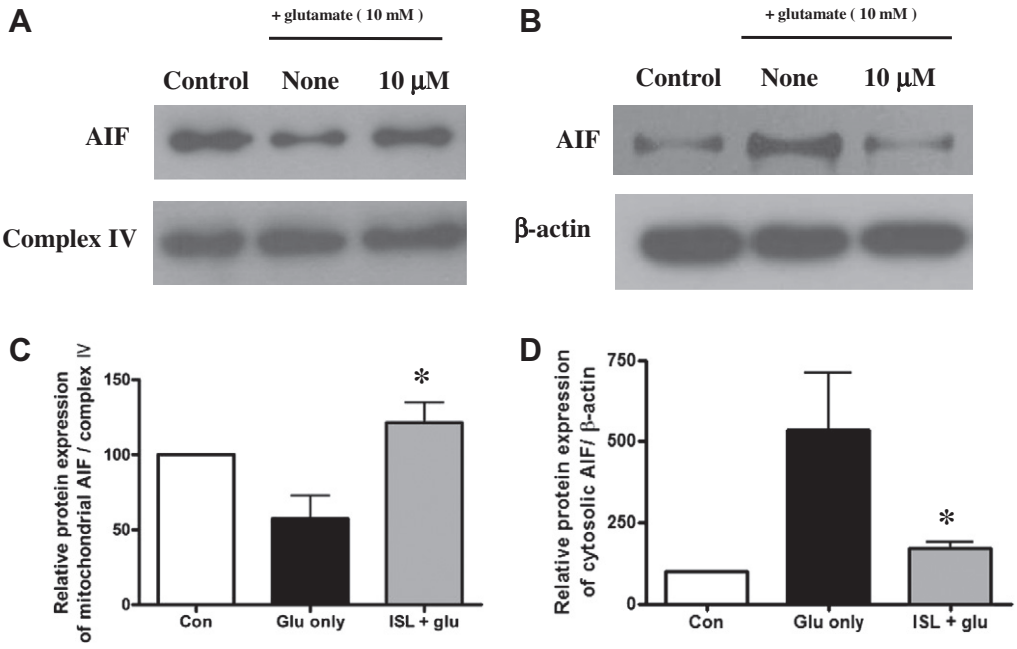

Fig. 4. ISL decreases the translocation of apoptosis-inducing factor (AIF). (A) Mitochondrial and (B) cytosolic AIF protein levels were determined by Western Blotting analysis. (C) The relative abundance of each band was quantified by intensity measurements of AIF in contrast to control of mitochondrial (ComplexIV) and cytosolic (β-actin) fraction, respectively. HT22 cells were treated with 10 mM glutamate for 24 h (Glu only), and without treated (Control), and pre-treated 10 mM ISL for 12 h before 10 mM glutamate-treatment for 24 h (ISL + glu). The adjusted mean ± SEM. ($n = 3$) of each band is shown above each blot. $^{*}P < 0.05$ compared to 10 mM glutamate-treated cells (ANOVA, Scheffé's test).

an increase in cell death, we investigated whether ISL prevents release of AIF from mitochondria. While no changes were found in whole-cell lysates (data not shown), mitochondrial AIF protein levels were decreased (Fig 4A). Western Blotting analysis of the cytosolic fraction of glutamate-treated HT22 cells showed increased AIF protein levels (Fig 4B). These results indicated that AIF is released from mitochondria into the cytosol in HT22 cells undergoing apoptotic cell death following exposure to glutamate. However, in cells treated with ISL, glutamate treatment did not significantly alter mitochondrial or cytosolic AIF levels (Fig. 4). These results indicated that release of AIF from mitochondria into the cytosol was suppressed by ISL.

4. Discussion

ISL is a flavonoid with a chalcone structure (Fig. 1A). The basic chalcone structure is two benzene rings linked through an α,β-unsaturated carbonyl group [9]. Many studies have demonstrated that chalcones have antiinflammatory [24], and neuroprotective effects [25]. Among the chalcones, there is growing interest in the biological effects of ISL, which has been shown to have antiinflammatory [26], and antioxidative [27] effects. Moreover, ISL has recently been reported to have neuroprotective effects. For example, it has been shown that activation of the gamma-aminobutyric acid (GABA) receptors has neuroprotective effects [28] and that ISL acts as a positive allosteric modulator of $GABA_A$ receptor that exhibits hypnotic effects [29]. ISL also also inhibits cocaine-mediated dopamine release by modulating $GABA_B$ receptor [30]. In addition, it was reported that a NMDA receptor antagonist has a neuroprotective potential [31] and that ISL is a novel NMDA receptor antagonist [32]. However, the mechanisms by which ISL protects neuronal cells from glutamate-induced apoptosis are not understood. Therefore, we investigated the effects of ISL on glutamate-induced apoptosis in HT22 cells.

Our results clearly indicated that glutamate-induced cell death involves mitochondrial dysfunction with characteristics of apoptosis. Pretreatment with ISL inhibited glutamate-induced apoptosis in HT22 cells in a concentration-dependent manner (Fig. 1B). In cells treated with glutamate, 10 μM ISL reduced and increased numbers of apoptotic and living cells, respectively, as assessed by annexin V/propidium iodide dual staining followed by flow cytometry (Fig. 1C). These results were in agreement with those of previous studies showing that flavonoids such as ISL protect HT22 cells from glutamate-induced neurotoxicity. Structurally, isoliquiritigenin 2′-methyl ether and sappanchalcone are very similar to ISL. However, while the methoxyl group at C-2′ could not protect neuronal cells from glutamate-mediated cytotoxicity, the galloyl group of the B ring was very important for protecting the cells. Numerous studies have indicated that flavonoids with chalcone structures, such as ISL, have the capacity to act as antioxidants [33]. Therefore, we suspect that the phenolic OH group of the A ring plays a major role in the protective antioxidant effects of ISL against glutamate-induced oxidative damage.

To better understand the mechanism by which ISL acting as an antioxidant protects cells from oxidative glutamate-induced neurotoxicity, we first examined whether it inhibits intracellular ROS production. As shown in Fig. 2A, ISL reduced glutamate-induced ROS production. Oxidative stress and mitochondrial dysfunction are common features of a large number of chronic and acute neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Huntington's disease [34,35]. During apoptosis, a decrease in the mitochondrial membrane potential facilitates the release from mitochondria of signaling molecules that drive apoptotic cell death [36]. The release of apoptotic signaling molecules is most clearly illustrated by translocation of AIF from mitochondria to the cytosol and the abnormal distribution of mitochondrial proteins, such as Bcl-2 and Bax, which initiate apoptosis.

Our results indicated that ISL prevented glutamate-induced loss of the mitochondrial membrane potential in HT-22 cells, possibly by suppressing ROS accumulation (Fig. 2). It is well known that glutamate reduced and increased mitochondrial Bcl-2 and Bax protein levels, respectively [7]. These results clearly indicated that

glutamate modulates apoptosis by inducing changes in antiapoptotic and proapoptotic factors. The glutamate-induced changes in Bcl-2 and Bax protein levels were reversed by 10 μM ISL (Fig. 3). The apoptotic pathway activated by glutamate treatment involved release of AIF from mitochondria. As shown in Fig. 4, pretreatment of HT22 cells with ISL blocked glutamate-induced changes in mitochondrial and cytosolic AIF levels. These results indicated that ISL suppressed the release of AIF from mitochondria into the cytosol. ISL appears to have a protective effect against mitochondrial dysfunction resulting from glutamate induced oxidative stress.

As the effects of ISL on glutamate receptor-mediated neuronal cell death were unclear, we examined the effects of ISL on glutamate-induced cell death in primary cortical neuronal cells. Glutamate increased the cell death rate in a concentration-dependent manner. Furthermore, ISL inhibited glutamate-induced NMDA receptor-mediated neuronal cell death in primary cortical neuronal cells (Fig. S3). Although further investigations are required, the neuroprotective effects of ISL observed in the present study may be due to its antioxidative effects.

In conclusion, we demonstrated that ISL isolated from Gu protects against glutamate-induced hippocampal neuronal cell death through antioxidative effects (Fig. S4). Thus, the ethanolic extract of Gu and ISL are expected to be useful in the development of new drugs for neurodegenerative diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.053.

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