

# Glycyrrhizin Attenuates Kainic Acid-Induced Neuronal Cell Death in the Mouse Hippocampus

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Glycyrrhizin (GL), a triterpene that is present in the roots and rhizomes of licorice (*Glycyrrhiza glabra*), has been reported to have anti-inflammatory and anti-viral effects. Recently, we demonstrated that GL produced the neuroprotective effects with the suppression of microglia activation and proinflammatory cytokine induction in the postischemic brain with middle cerebral artery occlusion (MCAO) in rats and improved motor impairment and neurological deficits. In the present study, we investigated whether GL has a beneficial effect in kainic acid (KA)-induced neuronal death model. Intracerebroventricular (i.c.v.) injection of 0.94 nmole (0.2  $\mu$ g) of KA produced typical neuronal death in both CA1 and CA3 regions of the hippocampus. In contrast, administration of GL (10 mg/kg, i.p.) 30 min before KA administration significantly suppressed the neuronal death, and this protective effect was more stronger at 50 mg/kg. Moreover, the GL-mediated neuroprotection was accompanied with the suppression of gliosis and induction of proinflammatory markers (COX-2, iNOS, and TNF- $\alpha$ ). The anti-inflammatory and anti-excitotoxic effects of GL were verified in LPS-treated primary microglial cultures and in NMDA- or KA-treated primary cortical cultures. Together these results suggest that GL confers the neuroprotection through the mechanism of anti-inflammatory and anti-excitotoxic effects in KA-treated brain.

**Key words:** glycyrrhizinic acid, KA, neuroprotection, anti-inflammation

## INTRODUCTION

The administration of kainic acid (KA), an excitatory amino acid L-glutamate analog, is known to induce typical epileptic behavior by mice in a dose-dependent manner [1, 2] and to cause neuronal degeneration in limbic structures, such as, the CA1 and CA3 regions of the hippocampus [3-5]. KA-induced hippocampal damage may be triggered directly, but it could also be caused by the hyperactivities of excitatory afferent pathways [2]. Consequently, the progress of neuronal death continues for several

days in CA1 and CA3 regions after treatment with KA. Previous studies have also shown that the delayed neuronal cell death occurred in CA1 and CA3 regions of KA-administered mice and is associated with the activations of astrocytes and microglia, with apoptotic neuronal death, and with the enhanced productions of inflammatory cytokines and reactive oxygen species (ROS) [6-8]. Therefore, neuronal cell death detected in mouse hippocampus shows characteristics of both acute and delayed cell death.

Licorice is a natural product that is used to treat liver disease in traditional Chinese medicine. Glycyrrhizin (GL) is extracted from licorice root and has been used in food industry as a flavoring additive. GL has been reported to have a variety of pharmacological effects, in particular, the anti-inflammatory effect of GL and its derivatives has been reported long time ago [9]. Recently, it has been reported that both GL and 18 $\beta$ -glycyrrhetic acid (18 $\beta$ GA, the metabolite of GL) decrease inflammatory

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response via phosphoinositide-3-kinase/Akt/glycogen synthase kinase-3 $\beta$  (PI3K/Akt/GSK3 $\beta$ ) signaling and glucocorticoid receptor activation, respectively [10]. Furthermore, GL and 18 $\beta$ GA inhibit intracellular ROS production, the inductions of proinflammatory cytokines (TNF- $\alpha$ , COX-2, and IL-1 $\beta$ ), and the activations of various transcription factors (NF- $\kappa$ B, PI3K p110 $\delta$ , p110 $\gamma$ ) in LPS-treated cells [11]. In addition, GL also inhibits angiogenic activities and the tumor growth in mice, and in the endothelial cells, it decreases the production of reactive oxygen species (ROS) and ERK activation [12].

Recently, accumulating evidences indicate that GL confers neuroprotective effects. Chergn et al. [13] reported that GL has a neuroprotective effect against glutamate-induced excitotoxicity in primary neurons, and Kao et al. [14] reported the neuroprotective effects of GL and 18 $\beta$ GA in PC12 cells. In addition, it has been shown that GL attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokine induction and HMGB1 secretion [15]. We have also reported that GL efficiently suppressed infarct formation in the postischemic rat brain after middle cerebral artery occlusion (MCAO) and that its neuroprotective effect is accompanied by improvements in motor impairment and neurological deficits, and by the suppressions of microglial activation and proinflammatory cytokine induction [16]. The purpose of this study was to investigate the neuroprotective effects of GL in a KA-induced epileptic animal model. Its anti-epileptic and neuroprotective effects were investigated by examining epileptic behavior and neuronal death, respectively, and the underlying protective mechanisms involved were examined in relation to its anti-inflammatory and anti-excitotoxic effects.

## MATERIALS AND METHODS

### Animals

Male BALB/c mice (25-30 g) were housed under diurnal lighting conditions and allowed food and tap water *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol used in this study has been reviewed by the INHA University-Institutional Animal Care and Use Committee (INHA-IACUC) on their ethical procedures and scientific care, and it has been approved (Approval Number INHA-110321-81). Animals were randomly assigned to a KA-treated, KA and GL-treated, or control group. At the start of the experiment, animals weighed 25-30 g and were 10 weeks old.

### Kainic acid administration

Intracerebroventricular (i.c.v.) injection of KA into brain was

previously described (Cho et al., 2003). Briefly, male BALB/c mice (25-30 g) were anaesthetized by the intraperitoneal (i.p.) injection of a 2:1 mixture (3.5  $\mu$ l/g body weight) of ketamine (50 mg/ml) and xylazine hydrochloride (23.3 mg/ml), and then placed on a stereotaxic apparatus (Stoelting Co, Wood Dale, IL). Four  $\mu$ l of saline solution containing KA (0.2  $\mu$ g) was then injected into the right lateral ventricle (stereotaxic coordinates in mm with reference to the bregma were AP, -2.0; ML, -2.9; DV, -3.7) [17] of mice at 0.5  $\mu$ l/min. After 5 min, the needle was removed over 3 min to minimize backflow. Mice were kept on a warm pad until awake.

### Assessment of seizure behavior

Mice were observed for 150 min after the KA injection and scored using the following scale (Sperk et al., 1985) [18]: (+1) arrest of motion; (+2) myoclonic jerk of head and neck with brief twitching movements; (+3) unilateral clonic activity, frequent focal convulsions, salivation; (+4) bilateral forelimb tonic and clonic activity, frequent focal convulsions; and (+5) continuous generalized limbic seizures with loss of postural tone, and death within 2 hrs.

### Treatment with GL

GL (Sigma, St. Louis, MO) was dissolved in 0.89% NaCl and administered intraperitoneal (i.p.). In total 300  $\mu$ l of GL-containing solution was injected 30 min before KA (0.2  $\mu$ g, i.c.v.) treatment.

### Sampling of protein

BALB/c mice were sacrificed by cervical dislocation. Then the hippocampus CA1 and CA3 were removed quickly and placed in ice-cold RIPA buffer (50 mM Tris-HCl (pH7.4), 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, and a complete mini protease inhibitor cocktail tablet (Roche, Basel, Switzerland)). After homogenization, lysates were centrifuged at 14,000 rpm at 4°C for 15 min and supernatant liquors were frozen at -20°C.

### Immunohistochemistry

Mice brains were fixed with 4% paraformaldehyde by transcardiac perfusion and post-fixed in the same solution overnight at 4°C. Fixed brain was incubated with 30% sucrose overnight and sections (30  $\mu$ m) were prepared by using a vibratome. Immunological staining was performed following standard procedure. Primary antibodies were diluted 1:200 for anti-NeuN antibody (MAB377, Chemicon, Temecula, CA) and anti-ionized calcium binding adaptor molecule-1 (Iba-1) (Wako Pure Chemicals, Osaka, Japan), and 1:150 for anti-GFAP antibody (DB Bioscience, San Jose, CA). After washing with PBS containing 0.1%

Triton X-100, sections were incubated with anti-mouse IgG (Vector Laboratories, Burlingame, CA) for anti-Neu N and anti-GFAP, or with anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for anti-Iba-1 in PBS for 1 hr at room temperature and visualized using the HRP/3,3'-diaminobenzidine (DAB) system. Numbers of NeuN-positive cells in 0.01 mm<sup>2</sup> (0.1×0.1 mm<sup>2</sup>) for CA1 or in 0.0225 mm<sup>2</sup> (0.15×0.15 mm<sup>2</sup>) for CA3 areas were obtained by counting 12 photographs, 3 photographs per experiment. The representative pictures were presented from three independent experiments.

### **Immunoblotting**

Fifty µg of proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel. After blocking with 5% non-fat milk for 1 hr, membranes were incubated with primary antibodies diluted 1:1,000 for anti-Cox-2 (Santa Cruz Biotechnology, Inc, Delaware, CA), anti-iNOS (Abcam, Cambridge, MA), anti-IL-1β (Merck Millipore, Billerica, MA), and anti-α-tubulin (Cell Signaling, Denver, MA) overnight at 4°C. The next day, membranes were detected using a chemiluminescence kit (Roche, Basel, Switzerland) using anti-rabbit HRP-conjugated secondary antibody (1:2,000, Santa Cruz Biotechnology).

### **Primary microglial culture**

Primary microglial cultures were prepared as previously described [19]. In brief, cells dissociated from the cerebral hemispheres of 2- to 13-day-old postnatal rat brains Sprague–Dawley strain) were seeded at a density of 1.2×10<sup>6</sup> cells/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% FBS (Hyclone, Logan, UT) and 1% penicillin–streptomycin (Gibco, Carlsbad, CA). After 2 weeks, microglia were then detached from the flask by mild shaking and filtered through a nylon mesh to remove astrocytes. After centrifugation (1,000×g) for 5 min, the cells were resuspended in a fresh DMEM supplemented with 5% FBS, and plated at a final density of 1×10<sup>5</sup> cells/well on a 24 multi-well culture plate. On the following day, cells were subjected to various treatments. BV2 cells were grown in DMEM supplemented with 1% penicillin, streptomycin and 5% FBS.

### **NO measurements**

Primary microglial cultures plated on 24-well plates (1×10<sup>5</sup> cells/well) were treated with lipopolysaccharide (LPS; 200 ng/ml) for 24 hrs. To measure the amount of NO produced by microglia, 100 µl of the conditioned medium was mixed with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethylenediamine), and incubated for 10 min at room temperature. Absorbances were measured at 550 nm using a

microplate reader.

### **Primary cortical culture**

Primary cortical cultures, including astrocytes and neurons, were prepared from embryonic day 15.5 mouse cortices and cultured as described previously by Kim et al. [20]. Dissociated cortical cells were plated at a density of approximately 4×10<sup>5</sup> cells per well (five hemispheres per 24-well poly(d-lysine)- and laminin-coated plate). Cultures were maintained without antibiotics in MEM containing 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. At day 7 in vitro (DIV 7), when astrocytes had reached confluence underneath neurons, cytosine arabinofuranoside was added to a final concentration of 10 µM, and cultures were maintained for 2 days to halt microglial growth. Fetal bovine serum and glutamine were not supplemented from day 7, and media were changed every other day after day 7. Cultures were used at DIV 12 to 14.

### **NMDA, KA, or glutamate treatment**

Primary cortical cells were treated with serum-free MEM or HEPES controlled salt solution (HCSS) containing 50 µM NMDA (Sigma, St. Louis, MO) for 10 min, 100 µM KA (Sigma, St. Louis, MO) for 12 hrs, or 100 µM glutamate (Sigma, St. Louis, MO) for 1 hr. The medium was then removed and replaced with fresh MEM, and cells were cultured for 24 hrs.

### **LDH assay**

Twenty four hrs after treating cells with NMDA (50 µM, 10 min), KA (100 µM, 12 hrs), or glutamate (100 µM, 1 hr) 50 µl aliquots of media and 50 µl of LDH assay reagent (Roche, Mannheim, Germany) were mixed in a 96-well plate and incubated for 15 min. Optical densities were measured using a 96-well plate reader at 490 nm.

### **Statistical analysis**

Statistical analysis was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test. All data are presented as means±SEMs and statistical difference was accepted at the 5% level.

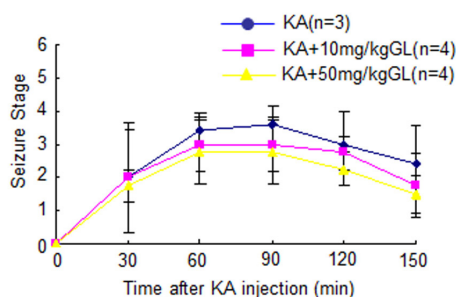
## **RESULTS**

### **GL treatment attenuated kainic acid (KA)-induced neuronal death in the hippocampus**

KA (0.2 µg, i.c.v.)-injected mice exhibited characteristic epileptic behavior as early as 5 min after treatment. Seizure progression from 0 to 150 min after KA administration was scored using

Sperk's seizure scale [18]. These fixed epileptic behaviors intensified rapidly and the highest level of reaction was observed 90 min after KA administration (Fig. 1). To examine the neuroprotective effect of GL, it was administered at 30 min prior to KA (10 or 50 mg/kg, i.p.) and seizure behavior was assessed at the same time points as in KA-administered control animals. Although the severity and duration of seizure behavior were generally lower in the GL-administered group than in KA-administered controls, differences were not statistically significant (Fig. 1).

It has been known that KA administration leads to neuronal death in CA1 and CA3 regions of the hippocampus. To determine degrees of neuronal death, brain tissue slices containing hippocampus were stained with anti-NeuN antibody at 2 days after KA treatment. In saline-treated control animals, mean numbers of NeuN<sup>+</sup> cells in the CA1 (0.1×0.1 mm<sup>2</sup>) and CA3

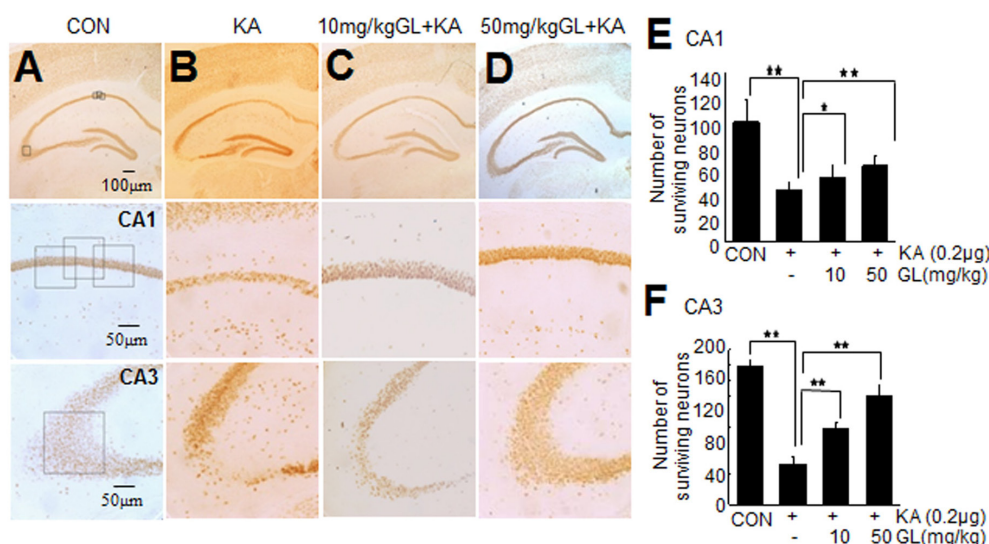


**Fig. 1.** No change in KA-induced epileptic behavior by GL. GL (10 or 50 mg/kg) was administered intraperitoneally (i.p.) 30 min prior to KA injection (0.2  $\mu$ g, i.c.v.). The seizure activity was scored using the rating scale devised by Sperk et al. [18]. Temporal seizure activities are presented as means $\pm$ SEMs (n=8).

(0.15×0.15 mm<sup>2</sup>) regions (indicated as black box in Fig. 2A) of the hippocampus were 99.3 $\pm$ 18.7 (n=12) and 178.7 $\pm$ 9.1 (n=12), respectively (Fig. 2A, E, F) and in the KA-administered controls, mean numbers of NeuN<sup>+</sup> cells were 42.9 $\pm$ 6.9 (n=12) and 51.7 $\pm$ 9.1 (n=12), respectively (Fig. 2B, E, F). When 10 mg/kg of GL was administered 30 min prior to KA, KA-induced neuronal death was significantly suppressed, and mean numbers of NeuN<sup>+</sup> cells were 53.5 $\pm$ 9.4 (n=12) and 97.2 $\pm$ 9.7 (n=12), respectively (Fig. 2C, D, F). KA-induced neuronal death was further suppressed by the administration of 50 mg/kg of GL and corresponding mean numbers of NeuN<sup>+</sup> cells were 63.0 $\pm$ 8.9 (n=12) and 140.8 $\pm$ 13.5 (n=12) (Fig. 2D, E, F). When GL (50 mg/kg) was administered 30 min after KA injection, KA-induced neuronal death was also significantly suppressed although the effects appeared to be slightly weaker than that observed in 30 min pre-administered animals (data not shown). These results indicated that GL suppressed KA-induced neuronal death in the mouse hippocampus.

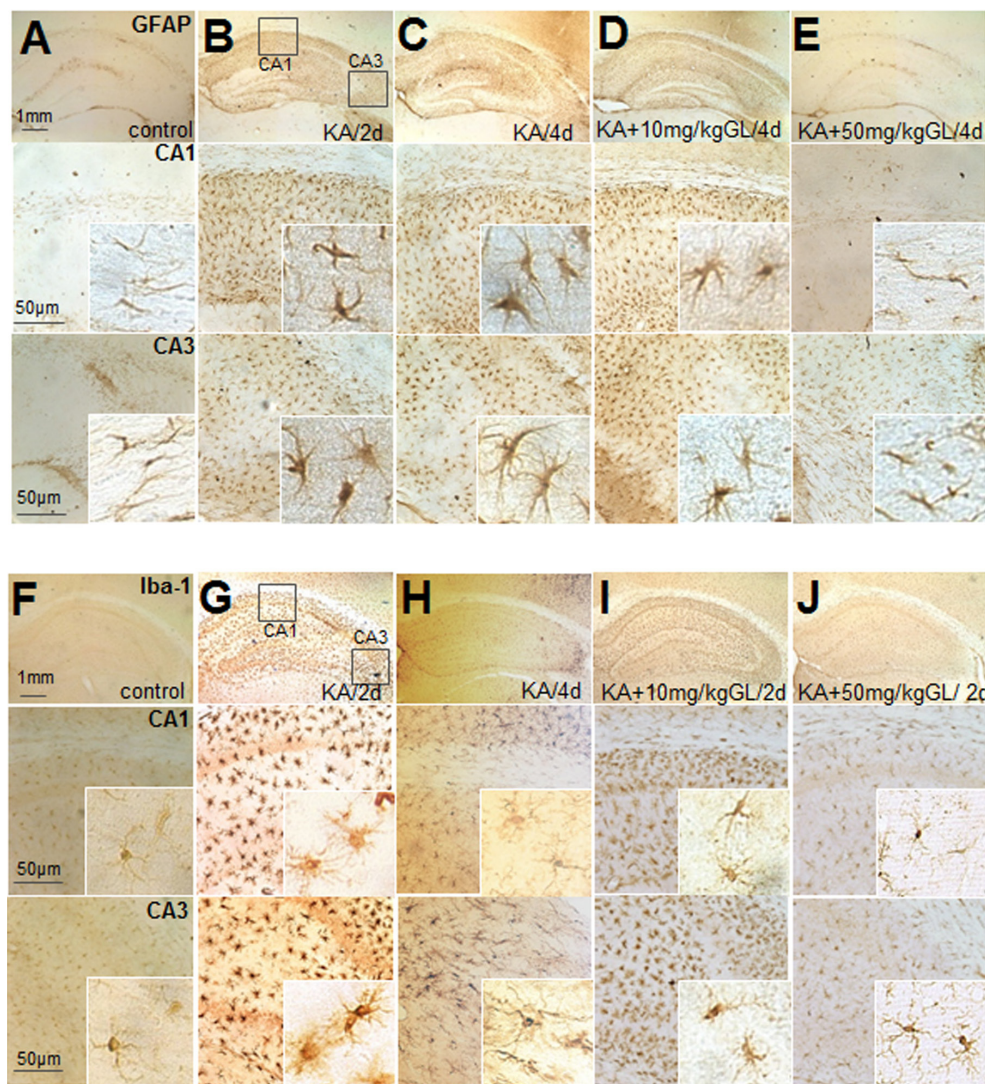
#### GL suppressed KA-induced astrocyte and microglia activations in CA1 and CA3

We examined whether GL affected inflammatory processes in KA-injected mice. Anti-GFAP and anti-Iba-1 antibodies were used to assess astrocyte and microglial activations, respectively. In saline-treated control animals, immunostaining with anti-GFAP antibody revealed that astrocyte cell bodies were small and their processes were long and thin (Fig. 3A). However, at 2 days after KA injection, astrocyte cell bodies were enlarged and their processes were shorter and thicker, that is, they possessed the characteristics of activating astrocytes (Fig. 3B). At 4 days after KA administration, astrocytes were activated further and exhibited the



**Fig. 2.** Attenuation of kainic acid-induced neuronal death in the hippocampus by GL. GL (10 or 50 mg/kg) was administered intraperitoneally (i.p.) 30 min prior to KA (0.2  $\mu$ g, i.c.v.). Hippocampal sections were prepared 2 days after KA administration and stained with anti-NeuN<sup>+</sup> antibody. Numbers of NeuN<sup>+</sup> cells were counted in the CA1 (0.01 mm<sup>2</sup>, 0.1×0.1 mm) and CA3 (0.0225 mm<sup>2</sup>, 0.15×0.15 mm) regions of the hippocampus (indicated as black boxes) and presented as means $\pm$ SEMs. \*p<0.05 and \*\*p<0.01. Scale bars represent 100  $\mu$ m in the upper panels of A-D and 50  $\mu$ m in the middle and lower panels of A-D.





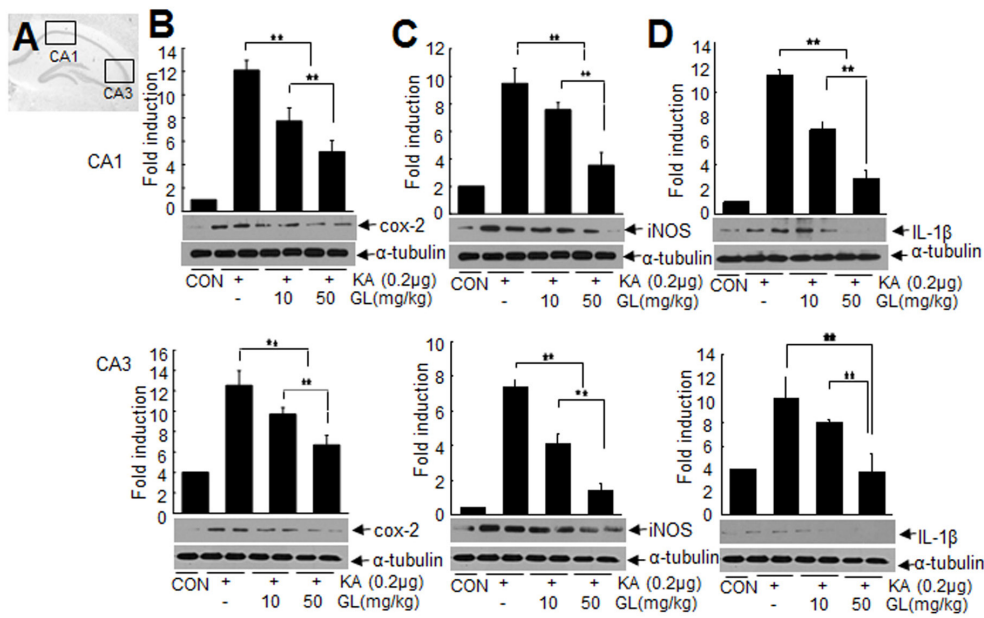
**Fig. 3.** Suppression of KA-induced astrocyte and microglia activations by GL. GL was administered (10 or 50 mg/kg, i.p.) 30 min prior to KA treatment (0.2 µg, i.c.v.). Hippocampal sections were prepared at 2 and 4 days post-KA treatment (0.2 µg, i.c.v.) and stained with anti-GFAP (A-E) or anti-Iba-1 (F-J) antibody. Photographs in middle and lower panels are high magnification micrographs of the CA1 and CA3 regions indicated as black boxes in B and G. The insets in each picture are high magnification micrographs of CA1 and CA3 regions. Representative picture from three independent experiments were presented. Scale bars represent 4 mm in the upper panels and 50 µm in the middle and lower panels.

characteristic star-shape of activated astrocytes (Fig. 3C). However, when 10 mg/kg GL was injected 30 min prior to KA, astrocyte activation at 4 days after KA administration was remarkably suppressed (Fig. 3D) and it was further suppressed by 50 mg/kg GL, adopting morphology of the resting state (Fig. 3E). Staining with anti-Iba-1 antibody (a marker of cells of myeloid origin [21]) showed that in saline-treated control animals, Iba-1-positive cells in the hippocampus were ramified (Fig. 3F). However, 2 days after KA injection, cell bodies of Iba-1-positive cells were enlarged and cytoplasmic processes were thicker and shorter in CA1 and CA3, displaying activated morphology of microglia (Fig. 3G). In contrast to astrocyte activation, staining with anti-Iba-1 antibody detected fewer number of Iba-1-positive cells exhibiting activated morphology at 4 days after KA administration (Fig. 3H). In 10 mg/kg GL-administered animals, number of Iba-1-positive cells exhibiting activated morphology was significantly suppressed at

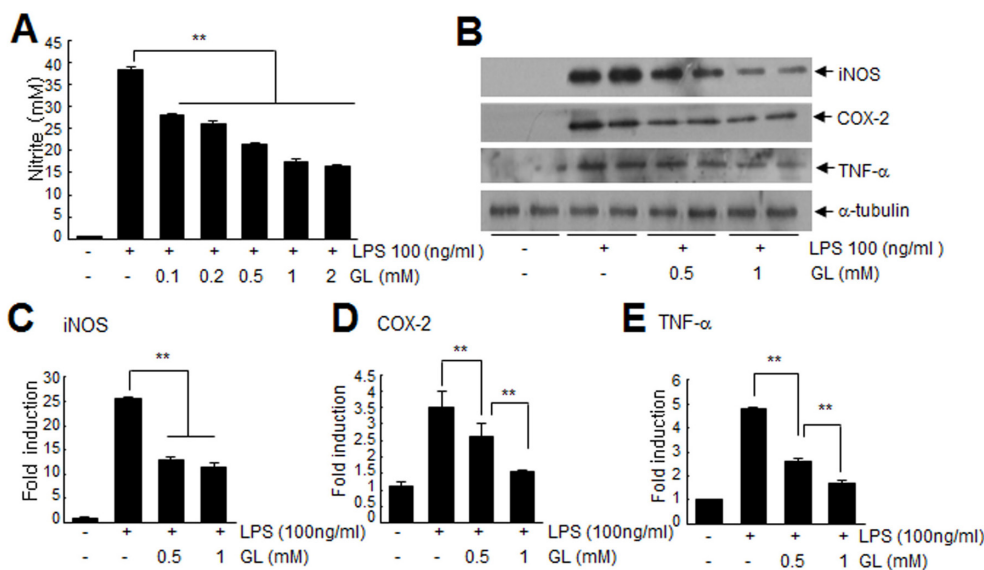
2 days post-KA (Fig. 3I) and it was almost completely suppressed by 50 mg/kg GL, wherein cell bodies of Iba-1-positive cells were thin and their cytoplasmic processes were ramified (Fig. 3J). These results suggest that the activations of microglia/macrophage and astrocytes in the hippocampus of KA-administered animals were suppressed by GL.

#### ***GL suppressed KA-induced proinflammatory marker production***

Next, we examined whether the inhibition of astrocyte and microglia activation by GL is accompanied by the suppression of proinflammatory marker production in the hippocampus. Levels of proinflammatory markers in KA-injected animals were assessed by immunoblotting with anti-COX-2, anti-iNOS, and anti-IL-1 $\beta$  antibodies. Protein samples were prepared from both CA1 and CA3 hippocampal regions (indicated in Fig. 4A) one day



**Fig. 4.** Suppression of KA-induced proinflammatory marker production by GL. GL (10 or 50 mg/kg, i.p.) was administered 30 min prior to KA (0.2 μg, i.c.v.) and levels of proinflammatory markers were assessed by immunoblotting. Protein samples were prepared at 1 day post-KA administration from the CA1 and CA3 regions (indicated in A) and results are presented as means±SEMs (n=4) \*\*p<0.01.



**Fig. 5.** Anti-inflammatory effects of GL in activated microglia. (A) Nitrite levels were used as a surrogate of NO levels. Primary microglia cultures ( $1 \times 10^5$  cells/well) were co-treated with 0.1, 0.2, 0.5, 1, or 2 mM of GL and 100 ng/ml of LPS for 24 hrs. Changes in nitrite levels are presented as means±SEMs (n=4). \*\*p<0.01. (B-E) Proinflammatory marker production was determined by immunoblotting at 24 hrs after treating cells with LPS (100 ng/ml) in the presence or absence of GL (0.5 or 1 mM). Representative pictures were presented (B) and results are presented as means±SEMs (n=4) \*\*p<0.01 (C-E).

after KA injection. COX-2 expression was notably increased both in CA1 and CA3 (Fig. 4B). After treatment with 10 mg/kg of GL, COX-2 expression was significantly suppressed and it was further suppressed by 50 mg/kg GL (Fig. 4B). iNOS and IL-1β expressions were also notably increased in KA-injected hippocampi and suppressed dose-dependently by GL (Fig. 4C, D). These results indicate that KA-induced proinflammatory marker productions were suppressed by GL.

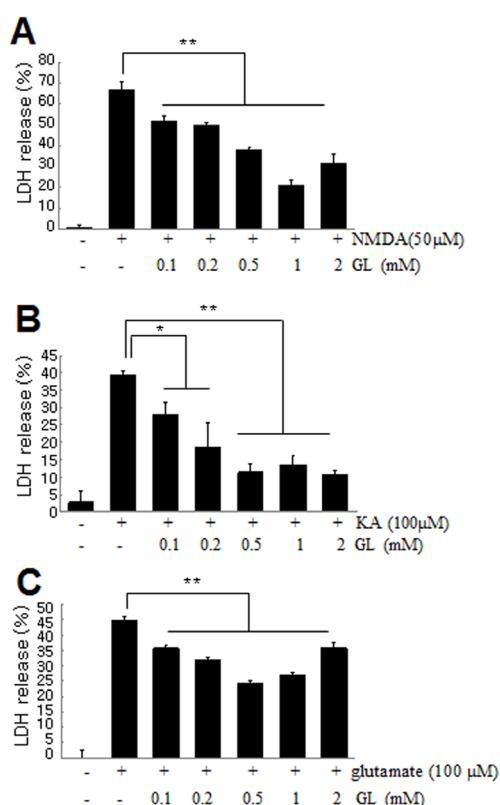
#### GL suppressed the LPS-induced activation of microglial cells

To investigate the anti-inflammatory effect of GL, the GL-

dependent suppression of microglial activation was examined in primary microglial cultures. Cells were stimulated with LPS (0.1 μg/ml) for 24 hrs with and without GL co-treatment, and nitrite production was measured. GL co-treatment reduced LPS-induced nitrite production dose-dependently; maximum inhibition was achieved at a GL concentration of 1 mM (Fig. 5A). In addition, GL dose-dependently repressed the inductions of proinflammatory markers (Cox-2, TNF-α, and iNOS) in LPS-treated primary microglial cultures (Fig. 5B-E). In contrast, α-tubulin levels were not changed at any GL concentration tested (Fig. 5B). These results indicate that GL suppresses LPS-induced microglial activation.

**Anti-excitotoxic effects of GL in primary cortical cultures**

GL-mediated suppression of neuronal death in KA-administered animal prompted us to examine an anti-excitotoxic effect of GL in primary cortical cultures. Since glutamate-mediated excitotoxic cell death is the main neurotoxic mechanism in epilepsy [22], NMDA, glutamate, and KA were used to induce excitotoxic damage in primary cortical cultures and to examine the anti-excitotoxic effects of GL. Severe neuronal damage was observed 2 hrs after NMDA treatment (50  $\mu$ M, 10 min) in primary cortical cultures (Fig. 6A). However, co-treatment with 0.5 mM of GL significantly reduced LDH release, and treatment with 1 mM of GL further reduced LDH release (Fig. 6A). Similar neuroprotective effects were also observed in KA (500  $\mu$ M, 12 hrs)- or glutamate (100  $\mu$ M, 1 hr)-treated primary cortical cultures (Figs. 6B, C), although, the protective efficacy of GL differed for the different treatments (Fig. 6). Together these results indicate that anti-excitotoxic effects in neurons contributes to the neuroprotective effects of GL in the postischemic brain.



**Fig. 6.** Effects of GL in NMDA-, KA-, or glutamate-treated primary cortical cultures. LDH levels in primary cortical cultures were measured at 24 hrs after NMDA (50  $\mu$ M, 10 min (A) or KA (150  $\mu$ M, 12 hrs (B), or glutamate (100  $\mu$ M, 1 hr) treatment in the presence or absence of GL (0.1, 0.2, 0.5, 1, or 2 mM). LDH levels are presented as means  $\pm$  SEMs. (n=4). \*p<0.05, \*\*p<0.01.

**DISCUSSION**

This study shows that GL has a strong neuroprotective effect in KA-administered mice. GL treatment was found to decrease neuronal death in the hippocampus, inhibited astrocyte and microglia activation in KA-administered mouse brain, and suppressed inflammatory marker induction. In addition, GL significantly suppressed NMDA-, KA-, or glutamate-induced neuronal death, indicating that GL confers anti-excitotoxic effect. In our previous studies, we have described inhibitions of microglia activation and concomitant inflammatory cytokine inductions by GL in the postischemic brain [16]. The present result, together with our previous report, suggests that neuroprotective effect of GL is, at least in part, attributable to its anti-inflammatory and anti-excitotoxic functions.

Recently, a number of studies have indicated that inflammation might be a consequence and also a cause of epilepsy [23]. It has been reported that inflammation contributes substantially to delayed brain damage after acute injury and that it detrimentally affects neurological outcome following KA-induced epileptic seizures [6,8]. In particular, astrocytes and microglia are activated and first upregulate proinflammatory cytokines (IL-1 $\beta$ , TNF, and IL-6) and cytokine receptor [24] and subsequently, an ensuing inflammatory process is induced not only in astrocyte and microglia but endothelial cells and peripheral immune cells migrated into the brain parenchyma. A numbers of studies have shown anti-inflammatory effects of GL in animal disease models, which is in accord with our findings. For example, GL was found to alleviate inflammatory lung disease, chronic obstructive pulmonary disease (COPD) [10], to reduce LPS-induced acute respiratory distress syndrome (ARDS) lethality [25], and to alleviate ovalbumin-induced allergic asthma in BALB/c mice [26]. Regarding the molecular mechanism underlying anti-inflammatory effect of GL, blocking the activations of NF- $\kappa$ B, PI3K, p110 $\delta$ , and p110 $\gamma$  has been reported [11]. In addition, inhibition of ROS formation and redox-sensitive signaling (nuclear factor- $\kappa$ B (NF- $\kappa$ B), Jun N-terminal kinase, and p38 mitogen-activated protein kinase) by GL has been shown to be responsible for suppression of pro-inflammatory gene expression [27]. Recently, the suppression of HMGB1 release by GL has also been reported to reduce apoptosis and inflammatory cytokines induction after transient spinal cord I/R injury [28] and in animal model of MCAO [16].

Although GL fails to inhibit proconvulsant effect of KA, we found that the anti-excitotoxic effect of GL might also contribute to its neuroprotective effect in a KA-induced seizure animal model (Fig. 6). It has been reported that in rat hippocampal slices and



primary neuronal cultures, GL can suppress glutamate-induced neurotoxicity and reduce cell death [13], which corroborates our results. In addition, carbenoxolon, a synthetic derivative of GL, has also been reported to block NMDA receptors and to impair long-term potentiation in mouse hippocampal slices [29]. It is of note that we also confirmed neuroprotective and anti-excitotoxic effects of carbenoxolon in stroke animal model and in KA-induced epilepsy animal model (Luo et al., unpublished data). Although, carbenoxolon has been known to function as a gap junction uncoupler [30], the mechanisms underlying the neuroprotective effects of GL need further study.

In terms of its neuroprotective potency, Hwang et al. [31] reported that the neuroprotective effect of roasted licorice against ischemic damage is greater than raw form and it might be through antioxidant effect. We have reported that SNMC, a glycyrrhizin-containing preparation developed for chronic liver disease, has a marked neuroprotective function in the postischemic brain via its anti-inflammatory effects [32] and SNMC shows significantly higher neuroprotective potency compared to an equivalent dose of pure glycyrrhizin, in terms of reducing infarct volume and improving neurological deficits [32]. Considering robust neuroprotective effects of GL in KA-induced animal seizure model in the present study and in MCAO animal model in our previous reports, investigation on the improvement of therapeutic potency of GL by modification and by producing formulation is worth pursuing.

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