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Neuroprotective effects of isoliquiritigenin against cognitive impairment *via* suppression of synaptic dysfunction, neuronal injury, and neuroinflammation in rats with kainic acid-induced seizures

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ABSTRACT

Epileptogenesis is a dynamic process initiated by insults to brain and commonly accompanied by cognitive impairment. Isoliquiritigenin (ISL), a flavonoid in licorice, has a broad spectrum of biological effects including anti-inflammatory and antioxidant activities. However, the protective effects of ISL against cognitive impairment in epileptic processes and the underlying molecular mechanism are not well understood. To address these questions, we established an reproducible seizure model by intracerebroventricular injection of kainic acid (KA) in 21-day-old rats; ISL was intraperitoneally administered three times prior to KA injection, and changes in cognitive function; synaptic plasticity; neuronal injury; number of glial cells; and expression of pro-inflammatory cytokines and nuclear factor-like (NRF)2 signaling and NACHT, LRR, and PYD domains-containing protein (NLRP)3 inflammasome components in the hippocampus were examined. Rats with KA-induced seizures showed longer average escape latency and decreases in the number of platform crossings and average time spent in the target quadrant in the Morris water maze; ISL pretreatment reversed this decline in cognitive impairment and increased the protein levels of synaptophysin, postsynaptic density-95 and brain-derived neurotrophic factor while reducing the number of Fluoro Jade B-positive cells, microglia, and astrocytes; cleaved-Caspase-3 and -9 protein levels; and tumor necrosis factor-α, interleukin (IL)-1β, and IL-18 production. It also enhanced the nuclear localization of NRF2, hemeoxygenase-1, and NAD(P)H:quinone oxidoreductase (NQO) 1, and reversed the upregulation of NLRP3 inflammasome components NLRP3 and Caspase-1 induced by KA injection. Thus, ISL protects against cognitive impairment in KA-induced epileptic processes possibly through regulation of NRF2 signaling and the NLRP3 inflammasome pathway.

1. Introduction

Epileptogenesis is a process leading to epilepsy originating from multiple neuropathologic lesions including seizure. Accumulating evidence indicates that the activation of early genes, neuronal death, sprouting, neurogenesis, and gliosis are involved in epileptogenesis [1–4]. Additionally, cognitive impairment is often observed in patients with epilepsy. Cellular and molecular events in epileptogenesis such as neuroinflammation, neuronal loss, and oxidative stress also contribute to cognitive impairment [5,6]. In fact, kainic acid (KA)-induced generalized seizure reduces cognitive function in rodents [7,8]; this serves as a model for exploring the molecular basis for cognitive impairment in epileptic disorders [9], which can potentially be treated by strategies that target neuroinflammatory mediators and neuronal injury.

Isoliquiritigenin (ISL) is a natural flavonoid from licorice (*Glycyrrhiza uralensis*) that has various biological effects including anti-

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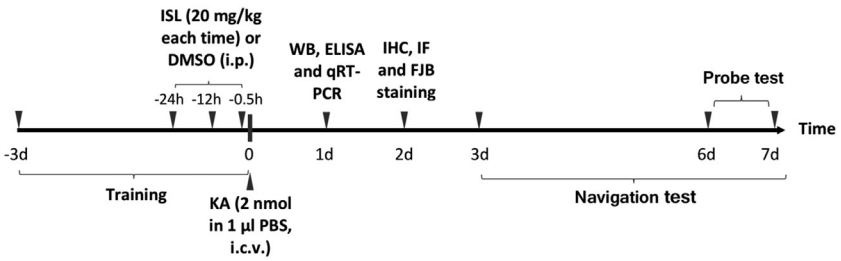

Fig. 1. Time schedule of the experimental procedures. DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FJB, fluoro jade B; i.c.v., intracerebroventricular; IF, immunofluorescence; IHC, immunohistochemistry; i.p., intraperitoneally. ISL, isoliquiritigenin; KA, kainic acid; qRT-PCR, quantitative real time (qRT)-PCR; WB, western blotting.

inflammatory, antioxidant, and antiallergenic activities [10,11]. ISL can readily permeate the blood-brain barrier into brain tissue [12]. ISL treatment was shown to alleviate early brain injury in experimental rodent models [13,14], and potently inhibits NACHT, LRR, and PYD domains-containing protein (NLRP)3 inflammasome activation while activating nuclear factor-like (NRF)2 [15–17]. As a member of the Nod-like receptor family, NLRP3 inflammasome plays an important role in the induction of the pro-inflammatory cytokine interleukin (IL)-1β. Blocking NLRP3 inflammasome activation attenuated intracerebral hemorrhage (ICH)-induced brain injury [18,19] whereas NLRP3 inflammasome activation was negatively regulated by NRF2 signaling [20]. ISL promotes the nuclear translocation of NRF2 and regulates the expression of antioxidant and detoxification enzymes and downstream proteins [13,17,21]. However, an early study reported that KA treatment failed to regulate NRF2 level in nuclear extracts of the hippocampus [22].

It remains unclear whether ISL has a protective effect against cognitive impairment in the early stages of epileptogenesis, and whether NRF2 signaling and the NLRP3 inflammasome are involved in this process. To answer these questions, we established a reproducible seizure model by intracerebroventricular (i.c.v.) injection of KA into 21-day-old rats as in our previous study [23]. KA administration induced progressive convulsive seizures culminating in status epilepticus (SE). We used this model to evaluate cognitive and underlying molecular changes in epileptogenesis and the therapeutic potential of ISL.

2. Material and methods

2.1. Animals and ethnic

Young male Wistar rats weighing 55 ± 3 g were obtained from the Experimental Animal Center, Shandong University. All rats were weaned at 18 days after birth and were housed in standardized environment condition with freely access to water and food and a 12 h alternating light and dark. Rats were subjected to stereotaxic surgery after acclimation. All experiments were made in accordance with the guidelines of the National Institutes of Health and the legal requirements in China. The experimental protocols and animal care were accredited by the Ethics Committee on Animal Experiments of the Second Hospital of Shandong University (No. KYLL-2014LW01). We made our efforts to minimize the number of animals used and their suffering.

2.2. Materials

KA was purchased from Sigma (St. Louis, MO, USA). ISL was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China) and dimethyl sulfoxide (DMSO) was provided by ZSGB-BIO (Beijing, China). Anti-ionized calcium-binding adapter molecule 1 (IBα1) antibody was purchased from Wako Chemicals (Richmond, VA, USA) and anti-glial fibrillary acidic protein (GFAP) antibody was obtained from Millipore (Bedford, MA, USA). Antibodies against neuronal nuclei (NeuN), synaptophysin, postsynaptic density (PSD)-95, brain-derived neurotrophic factor (BDNF), NRF2 and β-actin were purchased from Abcam (Cambridge, MA, USA); antibodies against cleaved-Caspase-3, cleaved-Caspase-9 were purchased from Cell Signaling (Beverly, MA, USA); antibodies against Caspase-1and Lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NLRP3 antibody and enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor (TNF)-α, IL-1β, and IL-18 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade.

2.3. Preparation of experimental animal model

Animal model of seizure was constructed by stereotaxic infusion of KA. Surgical operation was performed as described in our previous paper [23]. Briefly, rats were anesthetized and mounted on stereotaxic instrument. After the bregma being exposed, 2 nmol KA dissolved in 1 μl sterile phosphate-buffered saline (PBS, pH 7.4) was slowly injected into the right lateral ventricle. The stereotaxic coordinates were 0.6 mm posterior, 1.2 mm right, and 2.5 mm deep relative to the bregma [24]. Seizure activities of rats were being observed behaviorally and animals were returned to their cages after the cessation of behavioral seizures. All rats were randomly assigned into the subsequent experiments.

2.4. Experimental protocols

All animals were separated into four groups: normal group, ISL-only group, KA + vehicle (DMSO) group, and KA + ISL group. ISL (20 mg/kg) dissolved in DMSO was intraperitoneally administered at 30 min, 12 h and 24 h prior to KA injection. The same volume of vehicle was also administrated three times in KA + vehicle group. The rats of ISL-only group were injected by ISL alone. The dose of ISL was established by the results of preliminary experiments and published reports [13,25]. All rats, except those in behavioral tests, were anesthetized and sacrificed at 24 h and 48 h after KA injection for molecular experiments and histochemical experiments, respectively (Fig. 1).

2.5. Tissue sample preparation

For western blotting, ELISA and quantitative real time (qRT)-PCR experiments ($n = 6$ per group for each experiment), rats were anesthetized and brains were rapidly removed from the skull. The hippocampus was quickly dissected on ice and stored at −80 °C. For histochemical experiments (n = 6 per group), rats were perfused transcardially with PBS. And then, brains were removed and post-fixed overnight at 4 °C. Tissue samples were embedded with optimal cutting temperature compound. Coronal sections were obtained at the bregma level from −2.2 to −3.8 mm, cut at a thickness of 20 μm (1-in-6 series, 120 μm apart from each other), and stored at −20 °C.

2.6. Morris water maze (MWM) test

MWM test ($n = 10$ per group) was performed to evaluate the spatial memory ability as described previously [26], with slight modification. Briefly, the test consisted of a place navigation test and a spatial probe test, and was performed in a circular pool (120 cm diameter and 50 cm deep) filled with water at 25 ± 1 °C (Fig. 1). The pool was divided into four equal quadrants, and an escape platform (10 cm diameter and 1.0 cm beneath the surface of the water) was placed at the center of one

quadrant. A digital video camera was mounted above the pool, and swimming activity of the rats was recorded *via* video tracking software. Before the test, all rats were trained for 3 consecutive days before KA injection and rested for 2 days. The escape latency to find the platform was calculated for 5 consecutive days. In the place navigation test, each test was terminated when the rat reached to platform or after 60 s. On the 7th day after KA injection, the platform was removed for the probe test. Each rat was permitted to look for the platform in the pool for 60 s. The swimming speed, number of platform crossings and time spent in the target quadrant were recorded for further analysis.

2.7. Immunohistochemistry and immunofluorescence (IF)

Serial slides were used to detecting the immunoreactivity of IBα1 by avidin-biotin-peroxidase methods. Slides were incubated with rabbit anti-IBα1 antibody (1:500) overnight at 4 °C and then reacted with a biotinylated anti-rabbit IgG (1:500, Vector Laboratories Inc., CA, USA) for 1 h and avidin-conjugated peroxidase complex (1:200, Vector Laboratories) for 30 min at 37 °C. Positive reaction was observed using 3, 3′-diaminobenzidine tablet sets (ZSGB-BIO) and analyzed under the light microscope. For the immunoreactivity of NeuN and GFAP, slides were incubated with mouse anti-NeuN (1:200) or anti-GFAP (1:200) antibody overnight at 4 °C. After three washes, slides were reacted with Alexa Fluor 594-conjugated anti-mouse IgG (1:500, Invitrogen) for 1 h at room temperature. Slides were coverslipped and observed using a fluorescence microscope.

2.8. Western blotting analysis

Western blotting was performed following the manufacturer's instructions. Protein concentrations were measured by a BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China). Proteins (30 μg per lane) were separated in SDS-PAGE and analyzed using the following primary antibodies: anti-synaptophysin (1:1000), anti-PSD-95 (1:1000), anti-BDNF (1:1000), anti-cleaved-Caspase-3 (1:1000), anti-cleaved-Caspase-9 (1:1000), anti-NRF2 (1:1000), anti-NLRP3 antibody (1:1000), anti-Caspase-1 (1:500), anti-Lamin B (1:1000), and anti-β-actin (1:5000). After three washes, the membranes were treated with species-specific peroxidase-conjugated secondary antibodies for 2 h at room temperature. Specific immune bands were revealed with an Enhanced Chemiluminescence kit (Millipore). Values were normalized to the amount of β-actin or Lamin B in the same samples.

2.9. ELISA for TNF-α, IL-1β and IL-18

Hippocampal tissues were homogenized, and total protein was measured using a BCA protein assay kit (Beyotime Biotechnology). The levels of TNF-α, IL-1β and IL-18 were measured using appropriate ELISA kits. Results are described as pictogram per milligram total protein.

2.10. Fluoro Jade (FJ) B staining

After hydrated with alcohol series, slides were immersed in a solution of 0.06% potassium permanganate for 10 min and transferred to a solution of 0.0004% FJB solution (Millipore) for 20 min at 4 °C. After three washes, slides were coverslipped and examined with a fluorescence microscope.

2.11. Cell counting methods

Cell counting was performed according to our published method [23]. Briefly, six brain sections per animal were analyzed under a blinded manner at a 200× magnification. The number of target cells was counted twice on two differently visual fields of each hippocampal subfield including the hilus of dentate gyrus, cornu ammonis 3 (CA3) and CA1 subfield. The values of cells labeled by IBα1, GFAP and NeuN were expressed as the ratio of mean outcome in the experimental group to that in the normal group.

2.12. qRT-PCR for mRNA of hemeoxygenase (HO)-1 and NAD(P)H: quinone oxidoreductase (NQO) 1

Total RNA was extracted from dissected hippocampus using Trizol reagent (Invitrogen). First-strand cDNA was generated using the Reverse Transcription System (Qiagen, Valencia, CA, USA). qPCR was performed using Maxima SYBR Green dye (Fermentas, Glen Burnie, MD, USA) in an Eppendorf thermocycler (Hamburg, Germany). The primers were listed as follows; HO-1, forward: 5′-TGCTCGCATGAACA CTCTGGAGAT-3′, reverse: 5′-ATGGCATAAATTCCCACTGCCACG-3′; NQO1, forward: 5′- GTGAGAAGAGCCCTGATTGT-3′, reverse: 5′-CCTG TGATGTCGTTTCTGGA- 3′; GAPDH, forward: 5′-CCCTTCATTGACCTC AACTACA-3′, reverse: 5′-GCCAGTAGACTCCACGACA TA-3′. Each sample was run in triplicate. The expression of the target genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Gene expression was analyzed using the 2(−Delta Delta ct) method [27].

2.13. Statistical analysis

All data were expressed as means ± standard error of mean (SEM) and analyzed by SPSS version 21.0 software (SPSS, Inc., Chicago, IL, USA). The escape latency data in MWM test was analyzed using two-way analysis of variance (ANOVA) (group and time) with repeated measures (time) followed by Dunnett's test. All other data were analyzed with one-way ANOVA followed by least significant difference (LSD) test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ISL prevents cognitive impairment in rats with KA-induced seizures

The effect of ISL pretreatment on cognitive function in KA-induced seizure model rats was evaluated with the MWM test. The escape latency until the platform was located decreased in each group during the training trials. Rats in the KA + vehicle group spent more time finding the platform than normal rats from day 5 to 7. However, ISL pretreatment reduced escape latency compared to the KA + vehicle group on day 7 (Fig. 2a). In the probe trial, rats in the KA + vehicle group had fewer platform crossings compared with the normal group, whereas ISL pretreatment prior to KA injection increased the number of platform crossings compared with the KA + vehicle group (Fig. 2b). ISL pretreatment increased the time spent in the target quadrant relative to the KA + vehicle group (Fig. 2c). No significant differences were observed in escape latency, number of platform crossings, and time spent in the target quadrant between the normal and ISL-only groups. There was also no significant difference in the swimming speed among all groups (Fig. 2d).

3.2. ISL reverses synaptic dysfunction in rats with KA-induced seizures

Synaptic dysfunction is a key factor contributing to cognitive decline. We examined the effects of ISL pretreatment on the protein levels of the synapse-related proteins synaptophysin, PSD-95, and BDNF by western blotting (Fig. 3a). Synaptophysin and PSD-95 were downregulated in the hippocampus of the KA + vehicle as compared with the normal group, and ISL pretreatment reversed these effects (Fig. 3b-c). Similar trend was also observed in BDNF, ISL pretreatment significantly upregulated BDNP expression (Fig. 3d). These results indicate that ISL pretreatment protects against synaptic dysfunction induced by KA.

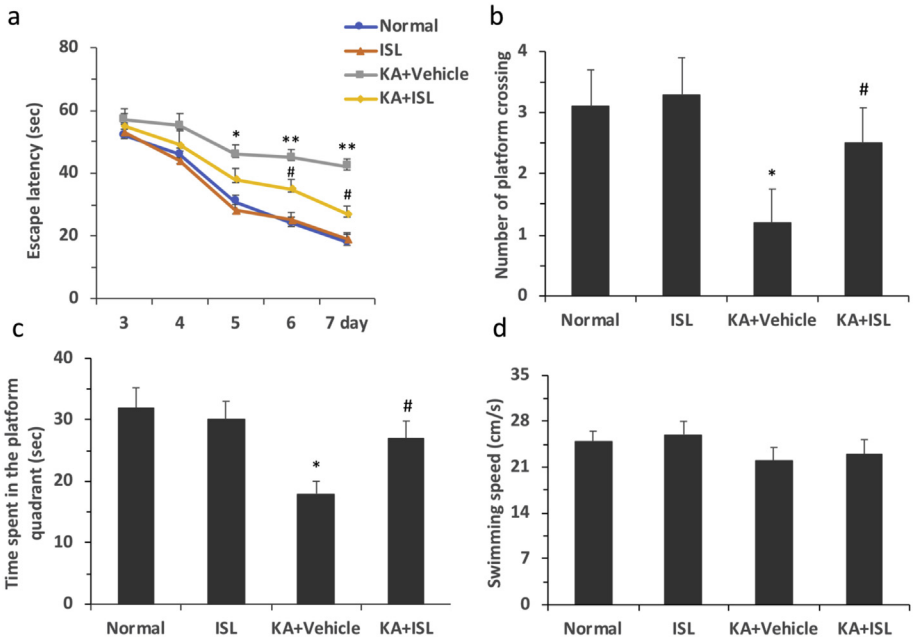

Fig. 2. ISL prevents cognitive impairment in rats with KA-induced seizures. Cognitive function was assessed by Morris water maze tests. Representative images show the escape latency (a), the number of platform crossings (b) and the time spent in the platform quadrant (c) and the swimming speed (d) in each group. Results are presented as mean ± SEM ($n = 10$). $^{*}p < 0.05$ *vs.* normal group, $^{\#}p < 0.05$ *vs.* KA + vehicle group.

3.3. ISL alleviates neuronal injury in the hippocampus caused by KA-induced seizures

To evaluate the effects of ISL pretreatment on neuronal injury, the expression levels of the apoptosis-related proteins cleaved-Caspase-3 and -9 in the hippocampus were evaluated by western blotting. Both proteins were upregulated in the KA + vehicle group as compared with the normal group; this was reversed by ISL pretreatment. No difference was observed between the normal and ISL-only groups (Fig. 4a-c). We also assessed neuronal injury in the hippocampus by IF and FJB staining. There was very little neuronal injury in the hippocampus of the normal group; however, there was significant neuronal injury in the KA + vehicle group. ISL pretreatment attenuated the KA-induced decrease in the number of hippocampal neurons. Similar results were observed with FJB staining; ISL pretreatment abrogated the increase in FJB-positive cells induced by KA relative to the KA + vehicle group (Fig. 4d-f).

3.4. ISL attenuates microglia and astrocyte activation and pro-inflammatory cytokine production

We measured the effects of ISL pretreatment on glial cell activation and the secretion of pro-inflammatory cytokines in the hippocampus. The microglia and astrocyte markers IBα1 and GFAP, respectively, were detected by immunohistochemistry. IBα1-labeled microglia had an amoeboid shape; moreover, protein expression was elevated in the KA-treated as compared to the normal group, which was abrogated by ISL pretreatment. Similarly, GFAP immunoreactivity—which was increased by KA—was reduced by ISL (Fig. 5a-c). Additionally, the increase in TNF-α, IL-1β, and IL-18 levels in the hippocampus of the KA + vehicle group relative to the normal group was abolished by ISL pretreatment, as determined by enzyme-linked immunosorbent assay (Fig. 5d-e).

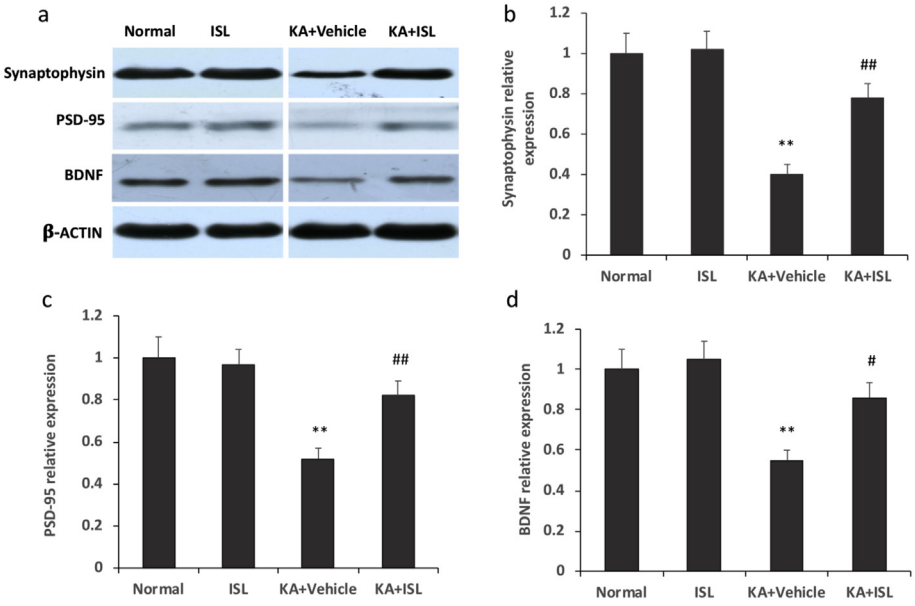

Fig. 3. ISL reverses synaptic dysfunction in rats with KA-induced seizures. (a-d) Western blotting and histograms show the protein levels of synaptophysin (b), PSD-95 (c) and BDNF (d) in the hippocampus of each group. β-Actin was used as a loading control. Results are presented as mean ± SEM ($n = 6$). $^{**}p < 0.01$ *vs.* normal group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* KA + vehicle group.

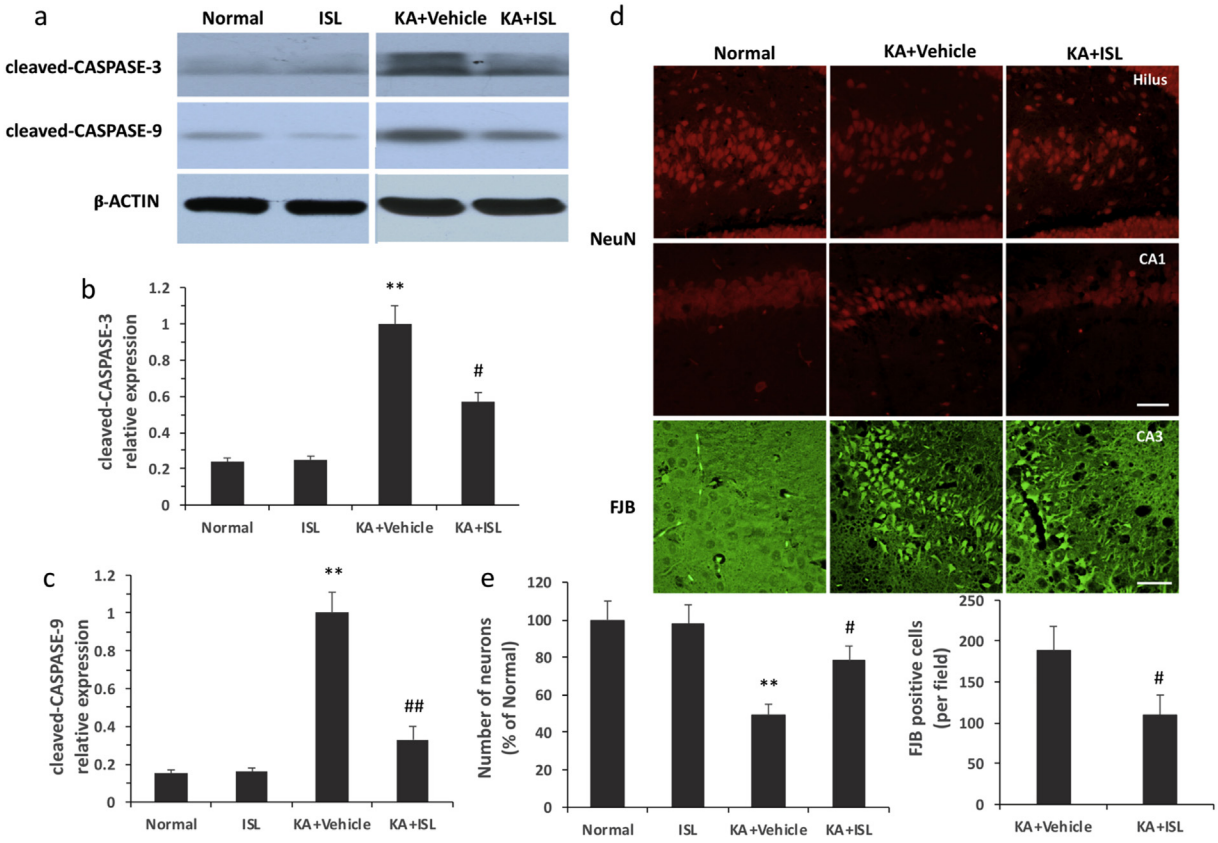

Fig. 4. ISL alleviates neuronal injury in the hippocampus caused by KA-induced seizures. (a-c) Western blotting and quantitative analysis show the protein levels of cleaved- Caspase-3 (b) and Caspase-9 (c) in each group. (d) Representative images show NeuN-positive cells in the dentate hilus and the cornu ammonis 1 (CA1) subfield of the hippocampus by IF staining, and FJB-positive cells in the CA3 subfield (scale bars = 50 μm). Histograms show the relative neuronal densities (e) the number of FJB-positive cells (f) in the hippocampus. Results are presented as mean ± SEM (n = 6). $^{**}p < 0.01$ *vs.* normal group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* KA + vehicle group.

3.5. ISL promotes NRF2 signaling and suppresses NLRP3 inflammasome activation

Nuclear localization of NRF2 in hippocampal neurons was increased in the KA + vehicle group as compared to the normal group, and was further promoted by ISL administration, as determined by western blotting (Fig. 6a-c). Accordingly, the mRNA upregulation of HO-1 and NQO1—cytokines acting downstream of NRF2—that was induced by KA was also significantly increased in the KA + ISL group (Fig. 6d-e). The levels of the NLRP3 inflammasome components NLRP3 and Caspase-1 were increased in the KA + vehicle group as compared to the normal group, but this trend was reversed by ISL pretreatment (Fig. 6f-h). These results suggest that ISL exerts protective effects against cognitive impairment in epileptic processes by mediating NRF2 signaling and NLRP3 inflammasome activation.

4. Discussion

The results of the present study demonstrate that ISL administered prior to induction of epilepsy by KA injection improved cognitive function—as evidenced by improved performance in the MWM test—and prevented the decrease in synapse-related proteins synaptophysin, PSD-95, and BDNF. ISL also reduced neuronal apoptosis and decreased the number of microglia and astrocytes as well as the production of pro-inflammatory mediators including TNF-α, IL-1β, and IL-18. We found that ISL also modulates NRF2 signaling and the NLRP3 inflammasome pathway *via* upregulation of nuclear NRF2 and its downstream effectors HO-1 and NQO1 and downregulation of the NLRP3 inflammasome components NLRP3 and Caspase-1. These results demonstrate that ISL exerts protective effects against cognitive impairment in epilepsy by improving synaptic plasticity, neuronal injury, and neuroinflammation.

The protective function of ISL in neurological diseases has been demonstrated by many studies. ISL administered at a concentration of 20 mg/kg alleviated behavioral deficits 24 and 72 h after ICH [13]. Consistent with earlier reports [7,8], our results show that i.c.v. administration of KA impaired performance in the Morris water maze but that ISL pretreatment reduced escape latency and increased the number of platform crossings and the average time spent in the target quadrant. In another study, ICR (a mouse strain from Institute of Cancer Research) mice fed a high-fat diet (HFD) and treated with ISL also spent more time in the target quadrant in the test [28]. These results provide evidence of the beneficial effects of ISL on cognitive function in a KA-induced epilepsy model.

Cognitive impairment is associated with lesions in specific brain areas and synaptic dysfunction. As such, the expression level of synaptic proteins such as the cytoskeletal components synaptophysin and PSD-95 can be used to assess cognitive ability [29–31]. In the current study, KA attenuated the expression of both proteins. Another trophic factor associated with cognitive improvement is BDNF, which was also downregulated in the hippocampus following KA administration as observed in stroke [32], spontaneous recurrent seizure [33], or Alzheimer's disease models [34,35]. ISL pretreatment reversed this effect. Liquiritigenin was shown to enhance the recovery of human immunodeficiency virus-1 Tat-mediated synaptodendritic injury *via* estrogen receptor [36]—which is also the receptor for ISL [37]—and alter depression-like behavior in mice *via* regulation of BDNF [38]. Thus, ISL pretreatment prevents cognitive decline by ameliorating synaptic

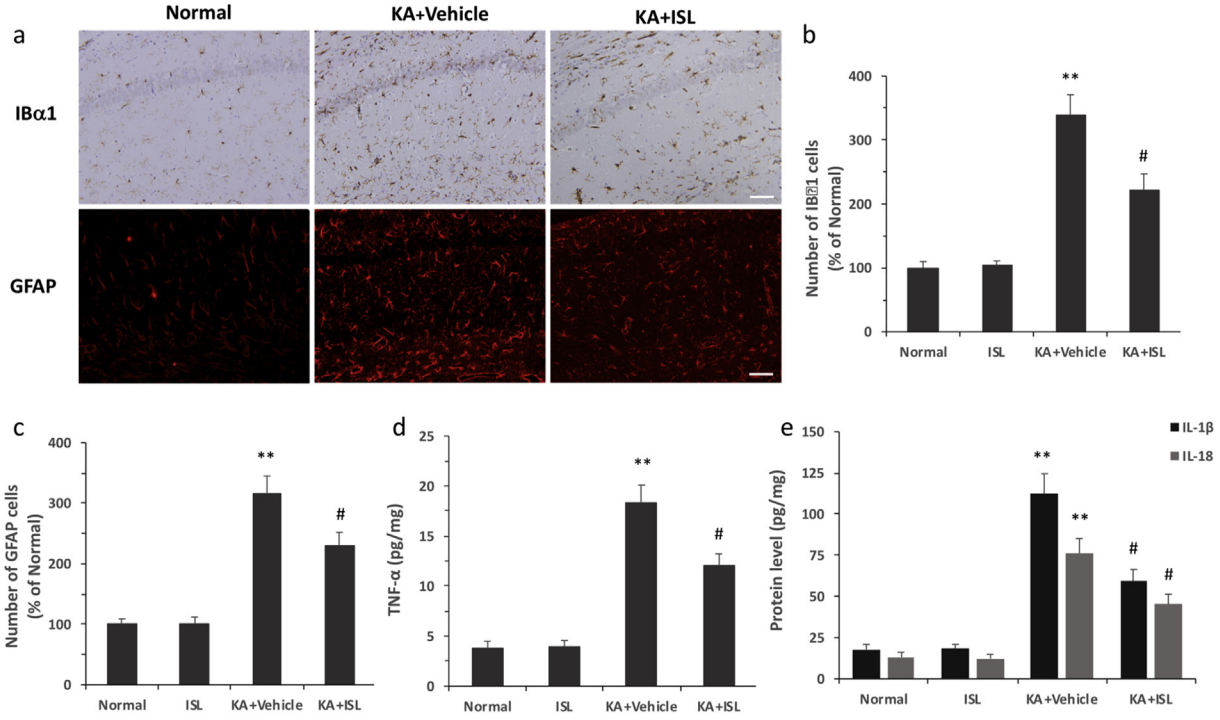

Fig. 5. ISL attenuates microglia and astrocyte activation and pro-inflammatory cytokine production. (a) Representative images show the immunoreactivities of IBα1-labeled microglia and GFAP-labeled astrocytes in the hippocampus of rats (scale bars = 50 μm). Counting analysis shows ISL pretreatment inhibited the increase of KA-associated IBα1-labeled microglia (b) and GFAP-labeled astrocytes (c). Quantitative analysis by ELISA demonstrates the protein levels of TNF-α (d), IL-1β and IL-18 (e) in the hippocampal homogenates. Results are presented as mean ± SEM (n = 6). $^{**}p < 0.01$ *vs.* normal group, $^{\#}p < 0.05$ *vs.* KA + vehicle group.

dysfunction in epilepsy.

Cognitive function depends on intact neurons. We previously described the neuronal injury that occurs in the hippocampal subfields in the early stages of epileptogenesis following SE induced by i.c.v. KA injection [23]. ISL treatment improved cerebral edema and neurological deficits in a mouse model of ICH [13,14]. Our results showed that neuronal injury following KA injection—as evaluated by IF and FJB staining—was blocked by ISL pretreatment, which also blocked the upregulation of cleaved-Caspase-3 and -9 in the hippocampus. Oxidative stress is a major contributor to neuronal apoptosis. KA induces oxidative damage in different areas of the brain; we speculate that ISL preserves cognitive function through anti-apoptotic effects [39,40], including restoring the balance of the oxidant-antioxidant system.

Neuroinflammation triggered by activation of glial cells and release of pro-inflammatory cytokines has a major impact on the pathogenesis of various neurological processes including cognitive impairment [5,41]. Clinical and experimental studies have shown that anti-inflammatory treatment can improve cognitive impairment. ISL administration was shown to reduce the number of myeloperoxidase-positive cells in perihematomal brain tissue of an ICH model [13], and blocked methamphetamine-induced GFAP expression in the striatum [42]. Our results showed that ISL pretreatment reduced the number of microglia and astrocytes and the production of TNF-α, IL-1β, and IL-18. Similarly, in ICR mice fed a HFD, ISL treatment reversed inflammation and insulin resistance by inhibiting the TNF-α/Janus kinase/insulin receptor substrate pathway [28]. Several *ex vivo* studies also found that ISL suppressed the production of nitric oxide, IL-6, TNF-α, and C−C motif chemokine ligand (CCL)2 in RAW264.7 macrophages [43–45]. Thus, ISL protects against cognitive impairment by decreasing inflammation.

NRF2 is a transcription factor that regulates cellular antioxidant responses. Under conditions of oxidative or xenobiotic stress, NRF2 is translocated to the nucleus, binds to antioxidant response elements (ARE), and regulates the expression of antioxidant and detoxifying enzymes such as HO-1, NQO1, and superoxide dismutase (SOD) [17,46]. Consistent with our results, published reports showed that KA could promote transient translocation of NRF2 to the nucleus [47], or enhance nuclear ARE binding [22], reflecting a defensive cellular response to the elevation of reactive oxygen species (ROS) induced by KA receptor stimulation. However, the functional NRF2 response still made numerous neurons succumb to the excitotoxic insult, which might be attributed to the discrepancies in the dose of neuroexcitatory agents [48], the vulnerability of hippocampal neurons to lesions [22,49], or the cellular types of NRF2 expression [50]. ISL may activate the expression of NRF2 and downstream target genes. In the ICH model, ISL treatment activated the NRF2-mediated antioxidant system, as indicated by the upregulation of NRF2 protein and SOD and chloramphenicol acetyltransferase activities, as well as nuclear translocation of NRF2 [13]. In the present study, upregulation of nuclear NRF2 and its downstream factors HO-1 and NQO1 following KA injection was further promoted by ISL pretreatment, indicating that the neuroprotective role of ISL is associated with its antioxidant activity, which involves modulation of NRF2 signaling. This was confirmed by the finding that ISL induced the expression of NRF2 downstream genes in NRF2 wild-type but not knockout mice [16]. As the best-characterized pattern recognition receptor, the NLRP3 inflammasome can detect diverse signals including pathogen-derived toxin nigericin, ROS, mitochondrial DNA, and amyloid-β [51,52]. Activated NLRP3 inflammasome cleaves Caspase-1, which triggers the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18. NLRP3 inflammasome components were upregulated in experimental models of brain injury [15,18]. In line with our results, a recent study reported that KA treatment stimulated the expression of IL-1β and NLRP3 in the hippocampus [53]. Inhibition of the NLRP3 inflammasome by small interfering RNAs, NLRP3 RNA interference, or chemical agents alleviated brain damage and cognitive impairment [13,53]; ISL inhibited the NLRP3 inflammasome and consequently, IL-1β production and Caspase-1 activation [15]. In our model, ISL pretreatment before KA injection attenuated the expression of NLRP3 inflammasome components,

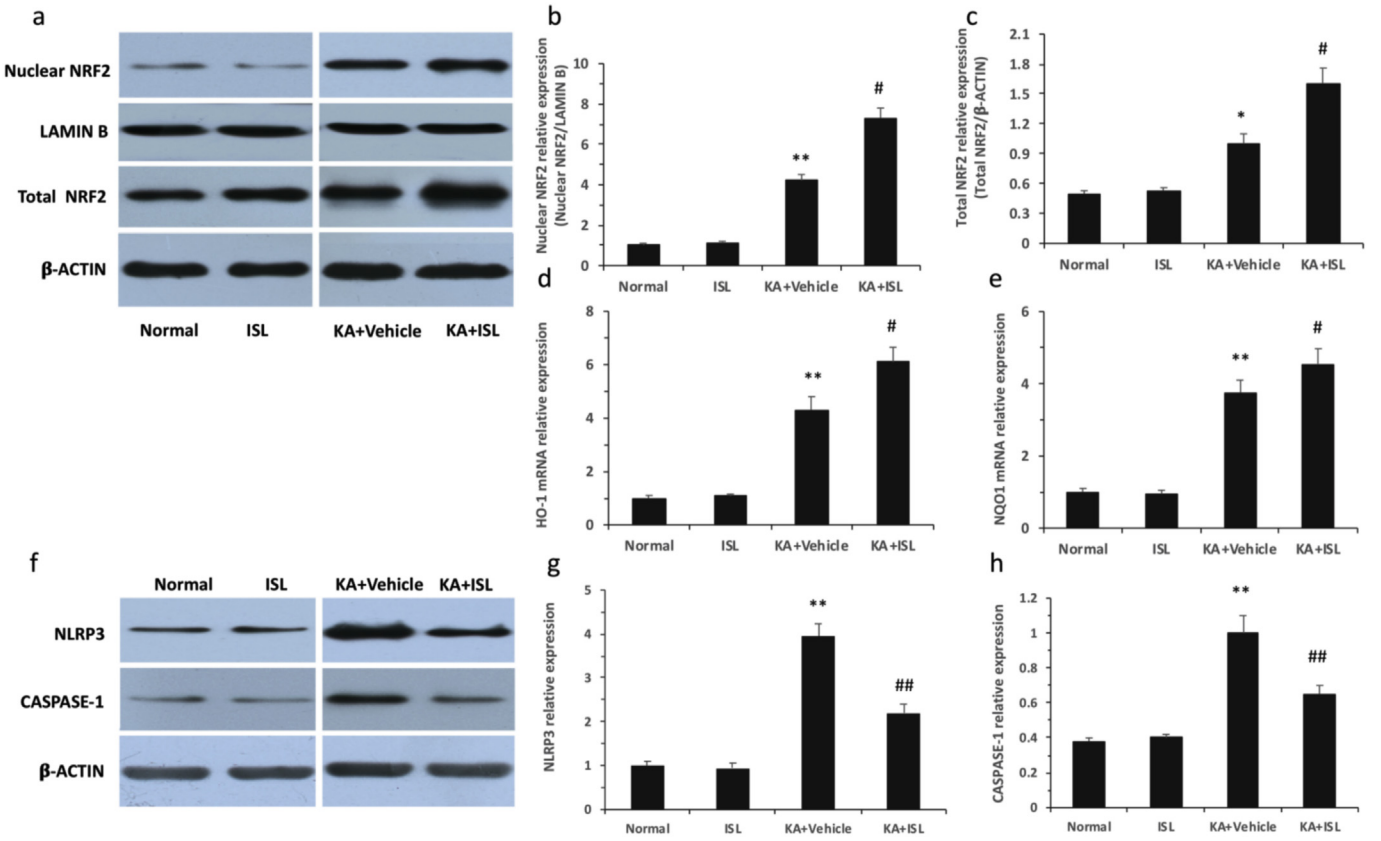

Fig. 6. ISL promotes NRF2 signaling and suppresses NLRP3 inflammasome activation. (a-c) Western blotting and histograms show the protein levels of nuclear NRF2 (b) and total NRF2 (c) in the hippocampus of each group. Lamin B and β-actin were used as a loading control. Quantitative analysis by qRT-PCR demonstrates the mRNA levels of HO-1 (d) and NQO1 (e) in the hippocampal homogenates. (f-h) Western blotting and histograms show the protein levels of NLRP3 (g) and Caspase-1 (h) in the hippocampus of each group. β-Actin was used as a loading control. Results are presented as mean ± SEM (n = 6). $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* normal group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* KA + vehicle group.

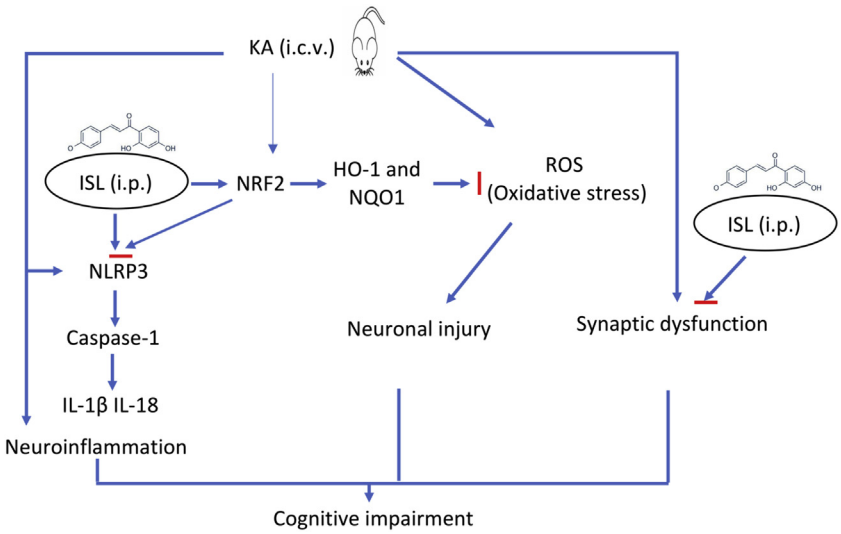

Fig. 7. Schematic diagram illustrates the underlying mechanism of ISL against cognitive impairment. HO-1, heme oxygenase-1; i.c.v., intracerebroventricular; IL, interleukin; i.p., intraperitoneally; ISL, isoliquiritigenin; KA, kainic acid; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; NQO1, NAD(P)H:quinone oxidoreductase1; NRF2, nuclear factor-like 2; ROS, reactive oxygen species.

including NLRP3, Caspase-1, and IL-1β and IL-18. Additionally, *in vivo and ex vivo* experiments demonstrated that NRF2 negatively regulates NLRP3 inflammasome expression and activity [13,15] and that ISL prevents neuronal apoptosis and neuroinflammation by stimulating nuclear translocation of NRF2 and inhibiting the NLRP3 inflammasome pathway (Fig. 7).

In summary, we found that ISL pretreatment improved cognitive function following KA-induced seizure by alleviating synaptic dysfunction and neuronal apoptosis and suppressing microglia and astrocyte activation and pro-inflammatory cytokine production. These effects are exerted *via* regulation of NRF2 signaling and the NLRP3 inflammasome pathway. These results provide evidence for ISL as a promising naturally derived agent for preventing cognitive decline in epilepsy and possibly other neurological disorders.

Conflict of interest

All the authors declare that there is no conflict of interests.

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