Original Research

Eugenol alleviates neuronal damage via inhibiting inflammatory process against pilocarpine-induced status epilepticus

Jing Zhu[1], Soojin Park[1], Chul Hoon Kim[2], Kyoung Hoon Jeong[3] and Won-Joo Kim[4]

[1]Department of Neurology, Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 03722, Republic of Korea; [2]Department of Pharmacology, Brain Korea 21 Project, Brain Research Institute, Yonsei University College of Medicine, Seoul 03722, Republic of Korea; [3]Epilepsy Research Institute, Yonsei University College of Medicine, Seoul 03722, Republic of Korea; [4]Department of Neurology, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul 06273, Republic of Korea
Corresponding authors: Won-Joo Kim. Email: kzoo@yuhs.ac; Kyoung Hoon Jeong. Email: jeongkh81@yuhs.ac

Impact Statement

Excessive inflammatory responses are a main pathological factor in various neurological diseases and are known to predispose or contribute to the development of epilepsy. Thus, the control of neuroinflammation may be important to develop disease-modifying strategies to prevent epileptic brain injury and epileptogenic progression. Eugenol, one of the essential oils extracted from several plants, possesses numerous beneficial properties such as antioxidant activity, but whether eugenol exerts anti-inflammatory effects to prevent epileptic brain injury remains unclear. This study showed that eugenol attenuates status epilepticus (SE)–induced apoptotic neuronal loss and reactive gliosis in the hippocampus. Moreover, eugenol suppresses SE-induced inflammatory processes including the production of pro-inflammatory cytokines, activation of nuclear factor-κB, and formation of inflammasome complex. These findings, for the first time, provide evidence showing the anti-inflammatory effect of eugenol for mitigating epileptic neuronal damage and suggest that eugenol may be a beneficial phytoconstituent for suppressing epileptogenic progression.

Abstract

Neuroinflammation is one of the most common pathological outcomes in various neurological diseases. A growing body of evidence suggests that neuroinflammation plays a pivotal role in the pathogenesis of epileptic seizures. Eugenol is the major phytoconstituent of essential oils extracted from several plants and possesses protective and anticonvulsant properties. However, it remains unclear whether eugenol exerts an anti-inflammatory effect to protect against severe neuronal damage induced by epileptic seizures. In this study, we investigated the anti-inflammatory action of eugenol in an experimental epilepsy model of pilocarpine-induced status epilepticus (SE). To examine the protective effect of eugenol via anti-inflammatory mechanisms, eugenol (200 mg/kg) was administrated daily for three days after pilocarpine-induced SE onset. The anti-inflammatory action of eugenol was evaluated by examining the expression of reactive gliosis, pro-inflammatory cytokines, nuclear factor-κB (NF-κB), and the nucleotide-binding domain leucine-rich repeat with a pyrin-domain containing 3 (NLRP3) inflammasome. Our results showed that eugenol reduced SE-induced apoptotic neuronal cell death, mitigated the activation of astrocytes and microglia, and attenuated the expression of interleukin-1β and tumor necrosis factor α in the hippocampus after SE onset. Furthermore, eugenol inhibited NF-κB activation and the formation of the NLRP3 inflammasome in the hippocampus after SE. These results suggest that eugenol is a potential phytoconstituent that suppresses the neuroinflammatory processes induced by epileptic seizures. Therefore, these findings provide evidence that eugenol has therapeutic potential for epileptic seizures.

Keywords: Eugenol, phytoconstituent, status epilepticus, hippocampus, neuroprotection, anti-inflammatory effects

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Introduction

Epilepsy is a common neurological disorder characterized by recurrent, unpredictable, and typically unprovoked seizures that affect approximately 65 million patients worldwide.[1] Status epilepticus (SE), one of the most severe manifestations of epilepsy, is a crucial medical emergency with high morbidity and mortality.[2] Many studies have shown that SE can have serious consequences, ranging from transient neurological dysfunction to life-threatening issues, and can trigger pathophysiological features, such as excessive glial activation, inflammatory responses, and irreversible neuronal damage.[3–5] In addition, pathophysiological changes induced by SE have been reported to contribute to the occurrence of recurrent seizures.[6,7] Accordingly, developing therapies to reduce post-SE brain injuries and the associated pathological features may be important to prevent epileptic progression.

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Clinical and experimental studies have verified that brain inflammation is the key mechanism underlying several neurological diseases.[8] In epilepsy, brain inflammation is both a cause and consequence of epileptogenesis. Studies have shown that inflammatory processes are characterized by the reactivity of glial cells, such as reactive astrocytes and activated microglia, in epileptic brain tissues, accompanied by severe neuronal damage.[9–11] As a brain injury factor, uncontrolled glial activation promotes the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), leading to neuronal cell loss and maladaptive synaptic plasticity.[12,13] Consequently, these inflammatory processes can trigger seizures and the development of epilepsy. Recently, several studies have shown that some anti-inflammatory drugs have a valuable therapeutic effect in patients with epileptic syndromes and in experimental models of epilepsy.[14,15] This evidence suggests that suppressing the inflammatory responses induced by acute seizure activity could be a crucial therapeutic strategy for preventing epileptic brain injuries, thereby inhibiting epileptogenic progression.

Eugenol (4-allyl-2-methoxyphenol) is one of the essential oils in plants including cloves, bay leaves, and various spices. Previous studies have shown that eugenol is traditionally used to treat dental caries and periodontal disease.[16] Moreover, it has been reported that eugenol possesses numerous properties, including antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant activity.[17] In the central nervous system, eugenol also provides neuroprotection against delayed neuronal death following ischemic damage[18] and prevents the toxic effects of amyloid-β peptides in PC-12 cells.[19] In epilepsy, studies have demonstrated that eugenol reduces seizure severity and neuronal excitability in experimental models of epilepsy.[20,21] This evidence suggests that eugenol can exert potential anticonvulsant activity; however, the effect of eugenol on excessive inflammatory responses following acute seizure has not yet been reported. In this study, we aimed to investigate whether eugenol treatment could alleviate neuropathological features via the mechanism of the anti-inflammatory effect of eugenol in the hippocampus after pilocarpine-induced SE.

Materials and methods

Pilocarpine-induced SE

A mouse model of pilocarpine-induced SE was established as previously described.[9] Briefly, adult male C57BL/6 mice (8 weeks old, 22–23 g, Orientbio, Gyeonggi, Korea) were housed under a 12-h light/dark cycle with food and water *ad libitum* for seven days. After an acclimation phase, to induce SE, mice were intraperitoneally injected with pilocarpine hydrochloride (325 mg/kg; Sigma, St. Louis, MO, USA), preceded by 30 min of scopolamine methyl nitrate (1 mg/kg, i.p.; Sigma) to reduce the peripheral effects of pilocarpine treatment. The sham-manipulated mice were injected with saline. The seizure stage was evaluated using the Racine scale,[22] and mice showing sustained severe seizure behaviors with generalized tonic–clonic seizures were considered to show SE; only these mice were used for this study. Two hours after SE onset, diazepam (10 mg/kg) was intraperitoneally injected to stop the seizure activity. Eugenol (200 mg/kg; Sigma), which showed the most effective concentration against brain damage, was dissolved in 40% β-cyclodextrin in distilled water, as previously described with some modification.[18,23] To evaluate the effect of eugenol treatment on SE-induced neuronal damage, eugenol or vehicle solution was treated by intraperitoneal injection per day, starting 1 h after diazepam treatment and continuing daily until three days after SE, as previously described.[9,24] The experimental mice were randomly divided into three groups: sham control (Sham; $n = 8$), mice treated with vehicle solution for three days after SE (SE3d-Veh; $n = 8$), and mice treated with eugenol for three days after SE (SE3d-EUG; $n = 8$). All procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at Yonsei University Health System and were performed in accordance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Fluorescent labeling for DNA fragmentation and quantitative analysis for apoptotic neuronal cell death

To prepare samples for cresyl violet staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and immunofluorescence staining, at three days after SE onset, mice were deeply anesthetized with 20% urethane in saline and were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). After perfusion, the brains were isolated and postfixed in the same fixative overnight at 4°C. Fixed brains were cryoprotected with 30% sucrose in 0.1 M PB for three days. As previously described,[9] serial coronal sections (20 μm thick) were collected between bregma – 1.46 mm and – 2.30 mm using a cryomicrotome (Leica Microsystems, Wetzlar, Germany). For histological assessment of hippocampal neuronal damage, we randomly selected the three sections for each animal, and then the selected sections were stained with 0.1% cresyl violet solution for 5 min. The sections were thoroughly dipped in absolute alcohol and covered with mounting solution. The stained sections were observed under a light microscope (BX51; Olympus; Tokyo, Japan). To evaluate apoptotic neuronal death, TUNEL staining was performed using a kit (Roche Diagnostics GmbH, Penzberg, Germany), as previously described.[24] The sections were incubated with the TUNEL mixture for 60 min at 37°C in the dark. After washing, the sections were mounted with 4′,6-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope (Axio Imager M2; Carl Zeiss, Thornwood, NY, USA). For quantitative analysis of TUNEL-positive apoptotic cell death in the hippocampus, three sections from each animal (between bregma – 1.46 mm and – 2.30 mm) were used to count TUNEL-positive cells in the CA1 and CA3 areas. To maintain consistency across animals, a rectangular box (1 mm × 0.5 mm) was centered over the hippocampal CA1 and CA3 regions, and the number of TUNEL-positive cells in each group was quantitatively expressed as previously described.[9]

Immunofluorescence staining for glial activation and imaging analysis

Immunofluorescence staining was performed as previously described.[24] In brief, using the same method as described above, the selected three sections were immersed in a blocking solution containing 5% bovine serum albumin in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature and then incubated overnight at 4°C with mouse antiglial fibrillary acidic protein (GFAP; MAB360; 1:500; Millipore, Temecula, CA, USA) and goat anti-ionized calcium-binding adapter molecule 1 (Iba1; ab5076; 1:500; Abcam, Cambridge, A, USA) antibody, followed by secondary Cy3-conjugated IgG (1:400; Jackson ImmunoResearch, West Grove, PA, USA) antibody. The sections were mounted and observed under a fluorescence microscope (Carl Zeiss). As previously described,[9,24] the immunoreactivity of GFAP and Iba1 was measured in the hippocampal subpyramidal regions between bregma – 1.46 mm and – 2.30 mm. Quantitative analyses were performed using a computerized analysis system and program (Image J; NIH, Bethesda, ML, USA).

Western blot analysis

To analyze the change in the levels of target proteins, brain tissues were prepared for western blot analysis, as previously described.[24] Isolated hippocampal tissues were homogenized with lysis buffer and centrifuged at 4°C for 15 min at 14,000*g*. The supernatants were transferred to fresh tubes, and the protein concentration was determined using a bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated using gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA) using an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated overnight at 4°C with specific primary antibodies: mouse anti-GFAP (1:1000; Millipore), goat anti-Iba1 (1:1000; Abcam), mouse anti-IL-1β (#12242; 1:1000; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-TNFα (#11948; 1:1000; Cell Signaling Technology), rabbit antiphosphonuclear factor-κB (p-NF-κB; #3033; 1:1000; Cell Signaling Technology), mouse anti-NF-κB (#6956; 1:1000; Cell Signaling Technology), mouse antinucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3; AG-20B-0014; 1:1000; AdipoGen, San Diego, CA, USA), mouse antiapoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC; AG-25B-0006; 1:1000; AdipoGen), rabbit anticaspase 1 (AG-20B-0042; 1:1000; AdipoGen), and mouse anti-β-actin (sc-47778; 1:4000; Santa Cruz Biotechnology; Dallas, TX, USA). After washing, the membranes were incubated with secondary antibodies (Enzo Life Science; Farmingdale, NY, USA), and the blots were developed with ECL western blotting detection reagents (Amersham Biosciences; Piscataway, NJ, USA). Band density was measured using a computer imaging device and accompanying software (Fujifilm; Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Inc.; Chicago, IL, USA) and Prism version 7 (GraphPad Software Inc.). The homogeneity of variance test was performed using Levene's test in SPSS Statistics. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple comparisons. Two-group analysis was performed using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. Data are displayed as mean ± standard error (SEM) with individual data points indicated or whiskers indicating the minimum and maximum values. Statistical tests and parameters are described in the figure legends.

Results

Eugenol protects SE-induced apoptotic neuronal damage in the hippocampus

Cresyl violet and TUNEL staining were performed to histologically assess the hippocampal neuronal damage following pilocarpine-induced SE. Treatment with eugenol alone revealed no adverse effect in the intact hippocampus (Supplementary Figure 1). Cresyl violet staining revealed severe neuronal damage in vehicle-treated animals, which showed many pyknotic cells in the pyramidal cell layer of the CA1 and CA3 subfields of the hippocampus three days after pilocarpine-induced SE, compared to the sham-manipulated groups (Figure 1(A)). In contrast, eugenol administration alleviated SE-induced neuronal damage in the pyramidal neurons of the CA1 and CA3 regions of the hippocampus (Figure 1(A)). In addition, the vehicle-treated SE groups revealed many TUNEL-positive cells in the pyramidal cell layer of the CA1 and CA3 subfields of the hippocampus after SE. However, eugenol treatment attenuated TUNEL-positive apoptotic neuronal death in the hippocampus following SE onset (Figure 1(B)). Consistently, the quantification of SE-induced apoptotic neuronal loss showed that eugenol treatment significantly decreased the number of TUNEL-positive cells in the CA1 ($P = 0.014$ vs SE3d-Veh) and CA3 subfields of the hippocampus ($P = 0.023$ vs SE3d-Veh) compared to that in the vehicle-treated SE groups (Figure 1(C)). These results suggest that eugenol protects hippocampal neurons from epileptic brain injury.

Eugenol attenuates reactive gliosis after SE

Next, we investigated whether eugenol treatment could mitigate glial activation, including reactive astrocytes and activated microglia, in the hippocampus following SE induction. Immunofluorescence staining with GFAP showed that SE markedly increased the expression of GFAP-positive astrocytes in the hippocampus, in contrast to sham-manipulated animals. However, eugenol administration attenuated SE-induced GFAP expression in the hippocampus (Figure 2(A)). When quantified by the immunoreactivity of GFAP expression, eugenol treatment significantly decreased GFAP expression in the subpyramidal area of the hippocampus compared to that in the vehicle-treated groups ($P < 0.001$ vs SE3d-Veh; Figure 2(B)). Consistent with the immunostaining results and quantitative analysis, western blotting showed a significant reduction in the protein levels of SE-increased GFAP expression by eugenol treatment ($P < 0.001$ vs SE3d-Veh; Figure 2(C) and (D)). Furthermore, eugenol treatment

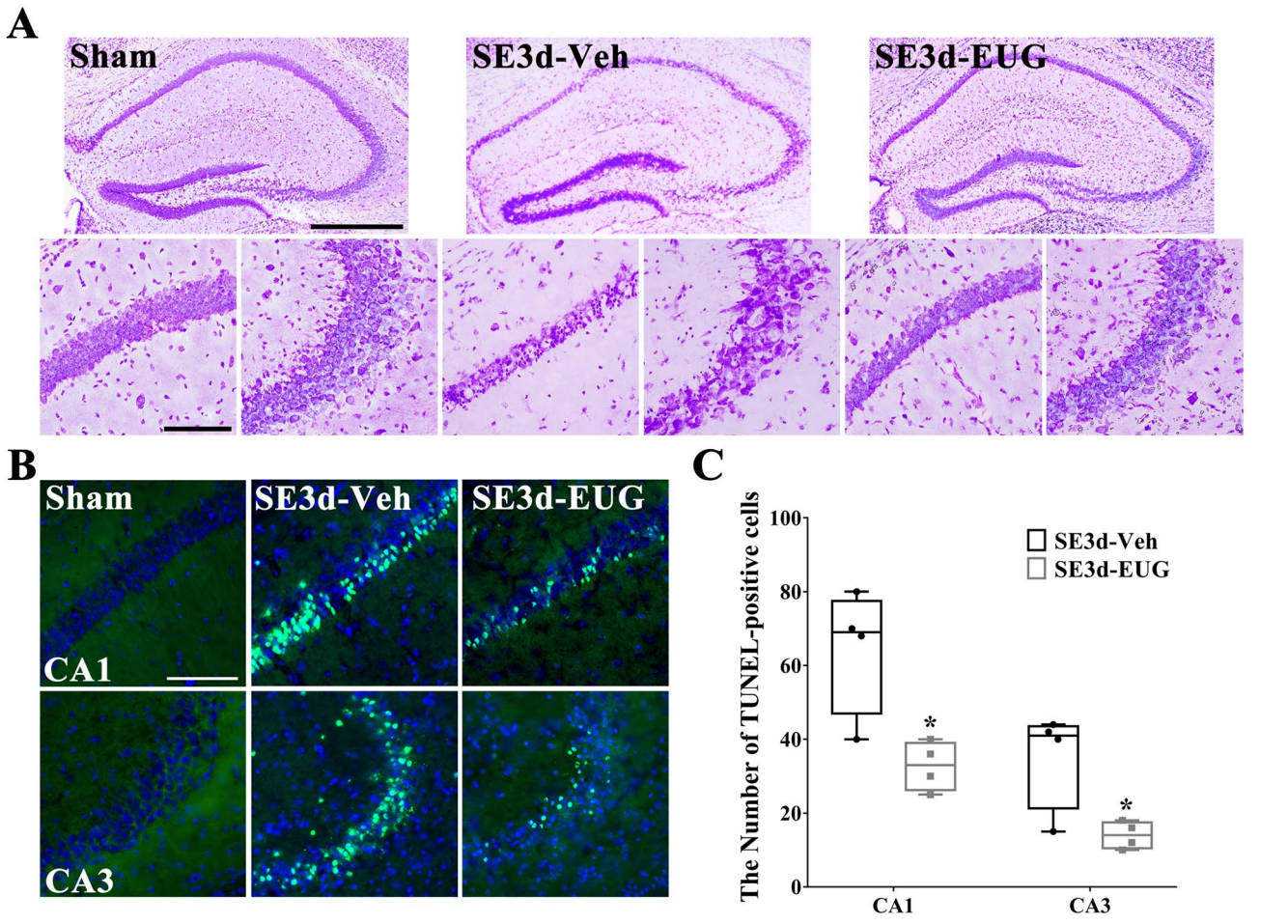

Figure 1. The protective effect of eugenol on neuronal damage in the hippocampus following pilocarpine-induced status epilepticus (SE). (A) Representative images show hippocampal cell death after SE measured by cresyl violet staining. In sham-manipulated hippocampus (Sham), intact neurons were found in the pyramidal neuronal layer of CA1 and CA3 regions. However, numerous pyknotic cells were observed in the CA1 and CA3 subfields three days after SE onset (SE3d-Veh). Compared with the vehicle-treated hippocampus (SE3d-Veh), eugenol treatment alleviated SE-induced neuronal loss in the CA1 and CA3 regions (SE3d-EUG). Scale bar = 500 μm; the same magnification was used in images for the hippocampus. Scale bar = 100 μm; the same magnification was used in images for CA1 and CA3 regions. (B) Representative images of the CA1 and CA3 pyramidal neurons labeled by TUNEL staining (green) in each group. Scale bar = 100 μm; the same magnification was used in all images. (C) Quantitative analysis shows that eugenol treatment significantly prevented SE-induced apoptotic neuronal death in the CA1 and CA3 regions, compared to SE3d-Veh groups. Whiskers represented minimum and maximum values.
Sham: sham-manipulated group; SE3d-Veh: mice treated with vehicle for three days after SE; SE3d-EUG: mice treated with eugenol for three days after SE.
*$P < 0.05$ versus SE3d-Veh (Student's *t*-test, $n = 4$ for each group).

significantly reduced the increased expression of Iba1, a marker for microglia/macrophages, in the hippocampus after SE (Iba1 immunoreactivity: $P < 0.001$ vs SE3d-Veh; the protein levels of Iba1: $P = 0.014$ vs SE3d-Veh; Figure 2(F) to (H)). These results suggest that eugenol treatment inhibits SE-induced reactive gliosis.

Eugenol reduces SE-induced pro-inflammatory cytokine production

Based on the above results, we demonstrated that eugenol treatment suppressed reactive gliosis in the hippocampus after SE. Considering the interconnection between glial activation and inflammation,[11,13] we next investigated the inhibitory effect of eugenol treatment on SE-induced inflammation. The levels of pro-inflammatory cytokines, such as TNFα and IL-1β, were examined by western blot analysis. We found that the elevated expression of TNFα and IL-1β in the hippocampal tissues of vehicle-treated SE groups was higher than that in the sham-manipulated groups (TNFα: $P < 0.001$ vs Sham; IL-1β: $P = 0.028$ vs Sham; Figure 3(A) and (B)). Treatment with eugenol significantly attenuated the levels of TNFα and IL-1β in the hippocampus compared with the increased protein levels in the vehicle-treated SE groups (TNFα: $P = 0.024$ vs SE3d-Veh; IL-1β: $P = 0.01$ vs SE3d-Veh; Figure 3(A) and (B)), suggesting that eugenol may exert anti-inflammatory properties in the epileptic hippocampus.

Eugenol inhibits the NF-κB activation and NLRP3 inflammasome expression after SE

To further investigate the effect of eugenol in SE-induced inflammation, we examined changes in NF-κB activation and the expression of NLRP3 inflammasome in the hippocampus after pilocarpine-induced SE. We found that pilocarpine-induced SE significantly increased NF-κB activation, as reflected by the ratio of p-NF-κB per NF-κB expression, in the hippocampus compared to that in the sham-manipulated

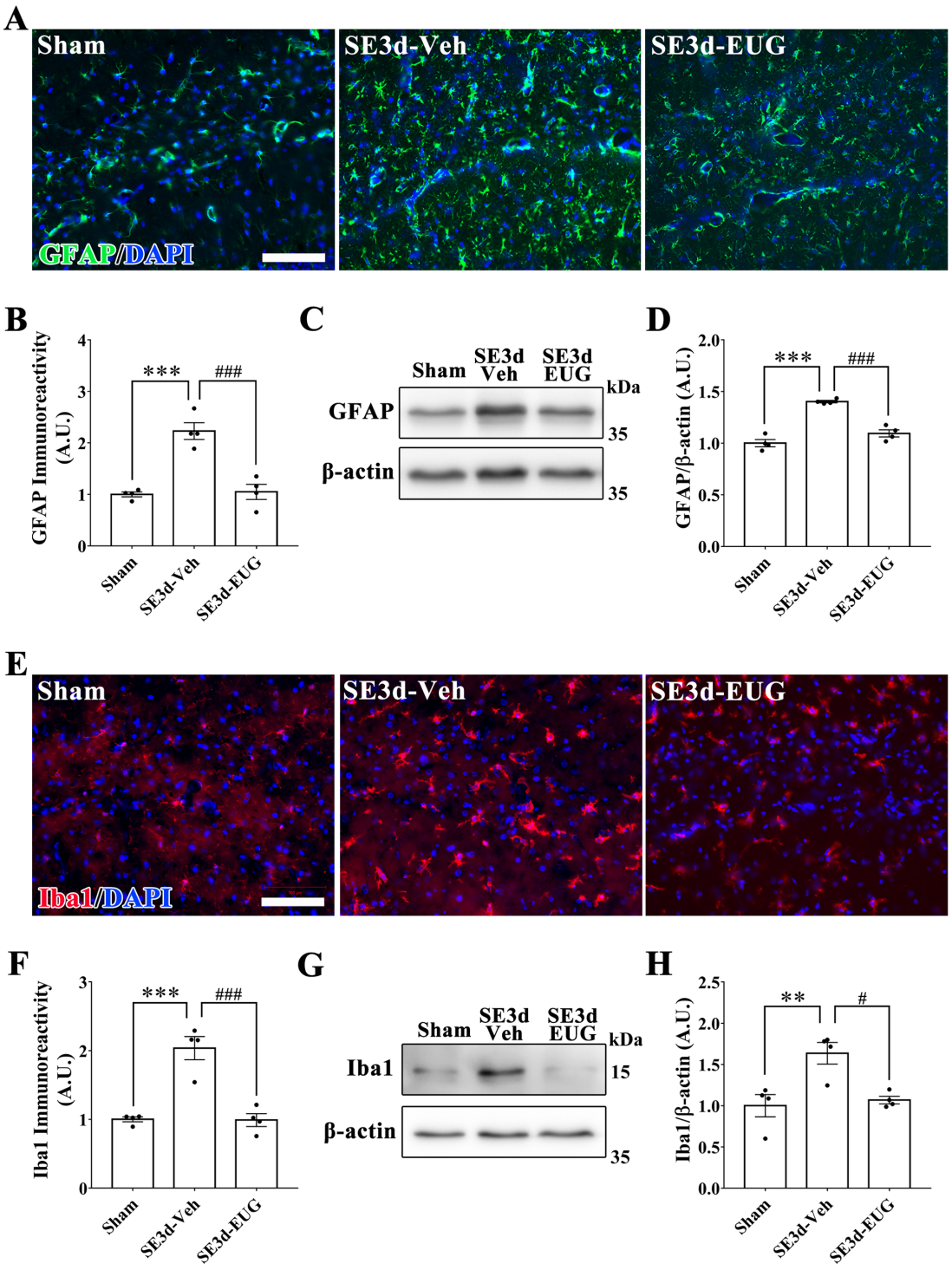

Figure 2. Inhibitory effects of eugenol treatment on SE-induced reactive gliosis in the hippocampus. (A) Representative immunofluorescence staining for GFAP-positive astrocytes (green) in the hippocampus after SE. Compared to the sham groups, many GFAP-positive astrocytes were found in the hippocampus of the SE3d-Veh groups; however, eugenol treatment mitigated SE-induced reactive astrocytes in the hippocampus (SE3d-EUG). Scale bar = 100 μm; the same magnification was used in all images. (B) The quantitative analysis of GFAP immunoreactivity is presented as the mean ± SEM with individual data points indicated. ***$P < 0.001$, Sham versus SE3d-Veh; ###$P < 0.001$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 28.94$, $P < 0.001$, $n = 4$ for each group). (C) Representative blots for GFAP expression in each group show that eugenol treatment significantly decreases the protein levels of GFAP expression after SE compared to those in the SE3d-Veh group. (D) Quantitative analysis based on the density of GFAP bands was normalized to β-actin expression. Data are presented as the mean ± SEM with individual data points indicated. ***$P < 0.001$, Sham versus SE3d-Veh; ###$P < 0.001$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 52.31$, $P < 0.001$, $n = 4$ for each group). (E) Representative immunofluorescence staining for Iba1-expressing microglia/macrophages (red) in the hippocampus after SE. Scale bar = 100 μm; the same magnification was used in all images. (F) Quantitative expression of Iba1 immunoreactivity. ***$P < 0.001$, Sham versus SE3d-Veh; ###$P < 0.001$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 28.32$, $P < 0.001$, $n = 4$ for each group). (G) Representative blots for Iba1 bands in each group. Note that eugenol treatment significantly reduces the protein levels of Iba1 after SE compared to those in the SE3d-Veh group. (H) Statistics of western blot analysis in Iba1 expression. Quantitative analysis based on the density of Iba1 bands was normalized to β-actin expression. Data are presented as the mean ± SEM with individual data points indicated. **$P < 0.01$, Sham versus SE3d-Veh; #$P < 0.05$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 9.763$, $P = 0.006$, $n = 4$ for each group).

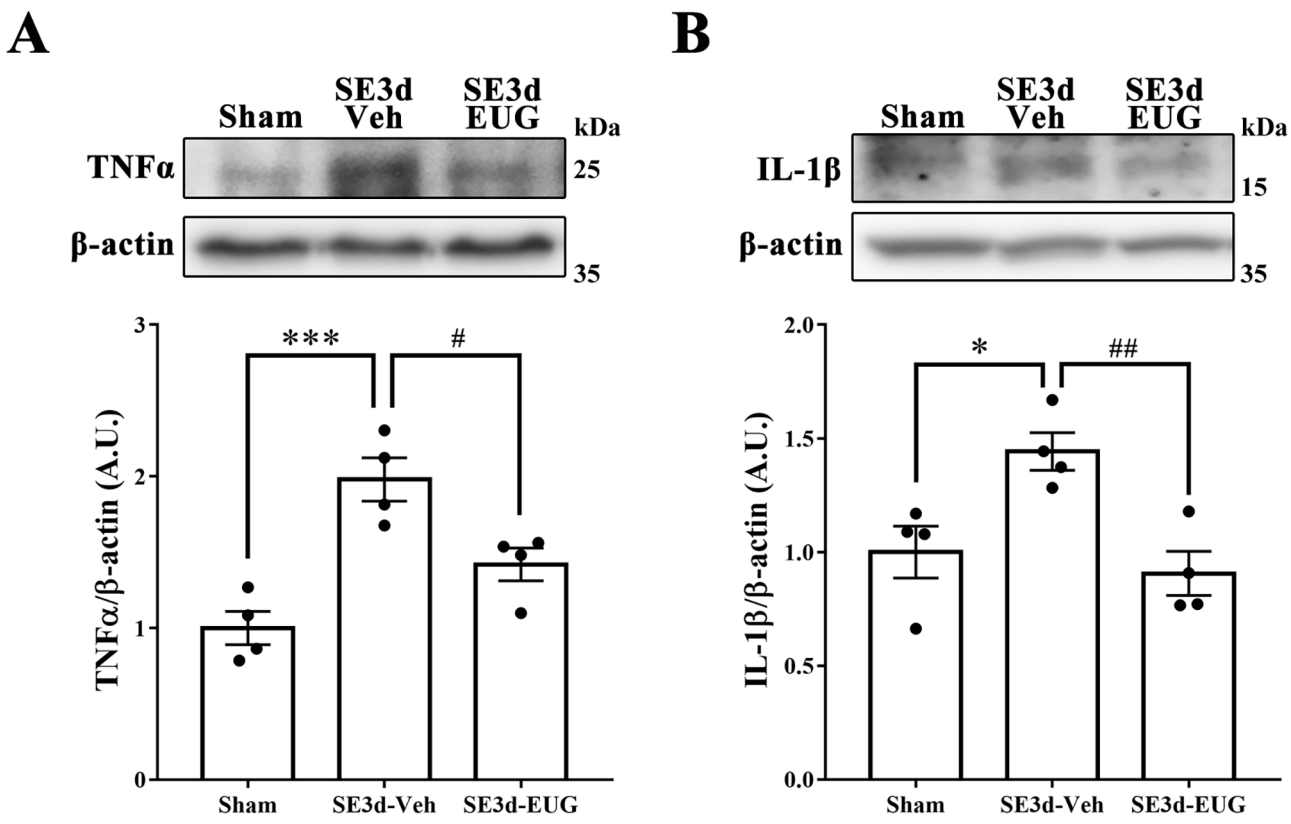

Figure 3. Decrease of SE-induced pro-inflammatory cytokines production following eugenol treatment. (A) Western blot analysis for TNFα expression shows that eugenol treatment significantly attenuated the elevated level of TNFα expression following SE onset compared to that in the SE3d-Veh group. Quantification of the TNFα protein bands was normalized to respective β-actin expression. Values are expressed as a mean ± SEM with individual data points indicated. ***$P < 0.001$, Sham versus SE3d-Veh; #$P < 0.05$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 16.43$, $P < 0.001$, $n = 4$ for each group). (B) Western blot analysis of IL-1β expression. Consistent with the result of TNFα expression, western blotting for IL-1β revealed a significant increase in the protein level of IL-1β in the group of SE3d-Veh, compared to that in the sham-manipulated group. In contrast, eugenol treatment induces a significant reduction in the level of IL-1β expression compared with the SE3d-Veh group. Quantification of the IL-1β protein bands was normalized to respective β-actin expression. *$P < 0.05$, Sham versus SE3d-Veh; ##$P < 0.01$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 8.438$, $P = 0.009$, $n = 4$ for each group).

groups ($P < 0.001$ vs Sham; Figure 4(A) and (B)), whereas eugenol treatment significantly reduced NF-κB activation compared to that in the vehicle-treated SE group ($P = 0.009$ vs SE3d-Veh; Figure 4(A) and (B)). Furthermore, eugenol treatment significantly suppressed the protein levels of the SE-induced inflammasome complex, including NLRP3, caspase 1, and ASC, in the hippocampus compared to that of vehicle-treated SE groups (NLRP3: $P = 0.002$ vs SE3d-Veh; caspase 1: $P < 0.001$ vs SE3d-Veh; ASC: $P = 0.041$ vs SE3d-Veh; Figure 4(A) and (C) to (E)). These results suggest that eugenol can reduce NF-κB activation and NLRP3 inflammasome-mediated pro-inflammatory pathology in the hippocampus following epileptic injury.

Discussion

The currently available antiepileptic drugs affect the relevant mechanisms for seizure initiation, propagation, and termination;[25] however, approximately 20–30% of patients with epilepsy cannot be controlled using any antiepileptic drugs.[26] Despite their apparent antiepileptic effects, a few drugs deteriorate neuronal damage or cognitive impairment.[27] Since it is still uncertain whether current antiepileptic drugs can improve the pathophysiological changes after epileptic seizures, there is a need for the broad insight of developing novel therapeutic strategies to prevent the epileptic brain injuries related to the occurrence of recurrent seizures. In recent years, globally, studies on phytoconstituents with antiepileptic effects have drawn much attention.[28] Previous studies have shown that eugenol exerts anticonvulsant effects against seizures induced by maximal electroshock or the administration of pentylenetetrazole.[29] Furthermore, previous studies have also shown that eugenol protected against neuronal loss by suppressing seizure-induced oxidative stress in the lithium-pilocarpine epilepsy model,[30] and suppressed the cytoarchitectural abnormality associated with epileptic progression in the hippocampal dentate gyrus following the kainic acid–induced SE.[23] These evidence suggest that eugenol may be a beneficial phytoconstituent for preventing neuropathological changes caused by epileptic seizures. Given these facts, we hypothesized that eugenol could also be useful for mitigating epileptic brain injury via its anti-inflammatory properties. The results of this study revealed that eugenol treatment attenuates apoptotic neuronal loss in the hippocampus after pilocarpine-induced SE. Eugenol treatment also reduced SE-induced reactive gliosis, and this effect was implicated in the suppression of inflammatory responses including the production of pro-inflammatory cytokines, NF-κB activation, and the NLRP3 inflammasome. Therefore, these findings indicate that the

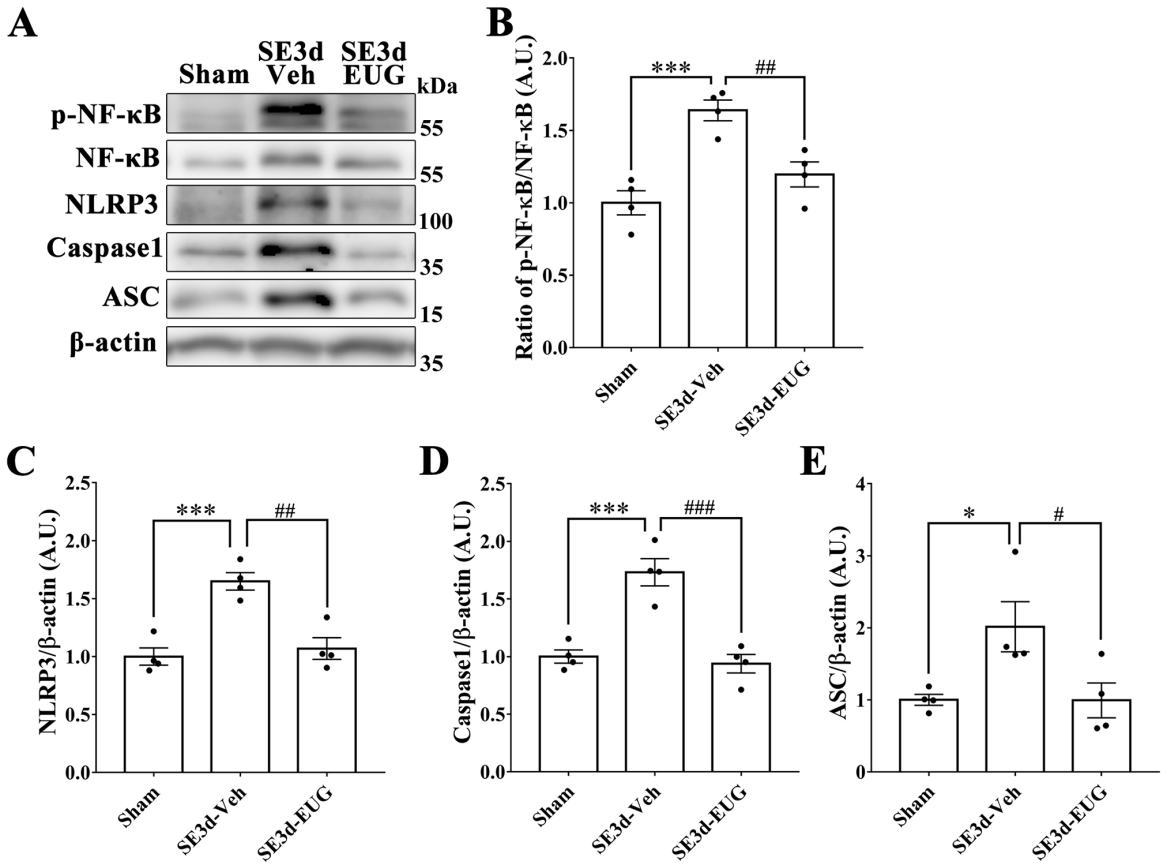

Figure 4. Reduction of SE-triggered NF-κB activation and NLRP3 inflammasome by eugenol treatment. (A) Representative blots of p-NF-κB, NF-κB, NLRP3, caspase 1, ASC, and β-actin expression in each group. Note that SE promotes the enhanced levels of p-NF-κB, NLRP3, caspase 1, and ASC in the hippocampus compared to those of sham-manipulated hippocampus, and eugenol treatment reduces these protein levels compared with SE3d-Veh group. (B) Histogram result shows quantitative analysis based on the ratio of p-NF-κB per NF-κB protein band normalized with the β-actin expression. Values are expressed as a mean ± SEM with individual data points indicated. ***$P < 0.001$, Sham versus SE3d-Veh; ##$P < 0.01$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 16.46$, $P < 0.001$, $n = 4$ for each group). (C) Histogram data of NLRP3 expression. ***$P < 0.001$, Sham versus SE3d-Veh; ##$P < 0.01$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 19.04$, $P < 0.001$, $n = 4$ for each group). (D) Quantification of caspase 1 expression. ***$P < 0.001$, Sham versus SE3d-Veh; ###$P < 0.001$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 24.57$, $P < 0.001$, $n = 4$ for each group). (E) Quantification of ASC expression. *$P < 0.05$, Sham versus SE3d-Veh; #$P < 0.05$, SE3d-Veh versus SE3d-EUG (one-way ANOVA with Tukey's post hoc test, $F(2,9) = 5.616$, $P = 0.026$, $n = 4$ for each group).

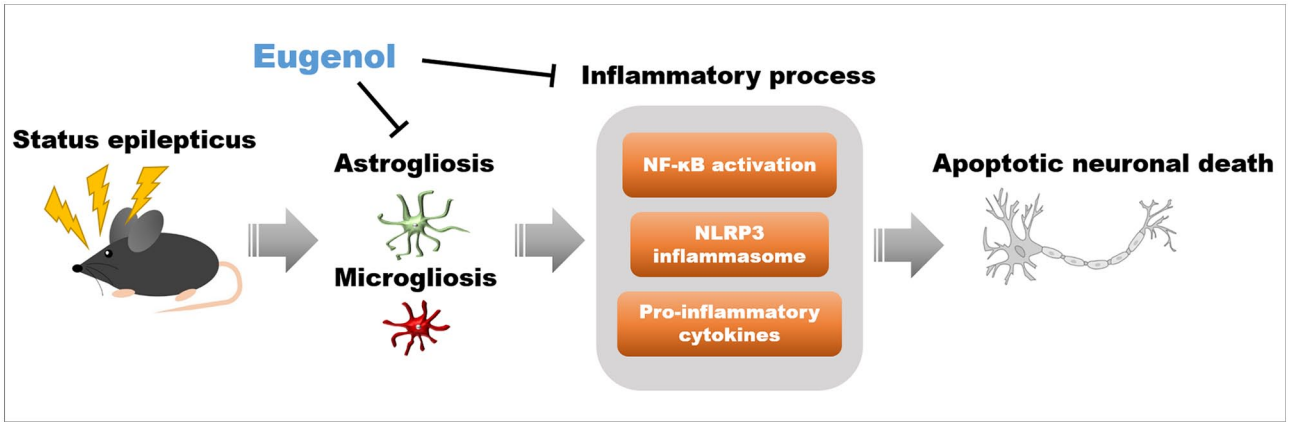

Figure 5. Schematic diagram showing the neuroprotective effects of eugenol. Eugenol treatment prevents apoptotic neuronal loss following SE induction by inhibiting reactive gliosis, and reducing pro-inflammatory cytokines production. These anti-inflammatory properties appear to be mediated through the suppression of NF-κB activation and NLRP3 inflammasome expression. Thus, eugenol may serve a potential therapeutic effect on epileptic brain damages.

neuroprotective effects of eugenol against epileptic brain injury may be attributable to inhibition of the inflammatory process (Figure 5).

Reactive gliosis has been well-established as one of the hallmarks of epileptic pathologies, including brain damage and neuronal cell loss post-SE induction.[31,32] Activated

astrocytes and microglia concomitant with SE promote the production of various pro-inflammatory cytokines, such as TNFα and IL-1β.[33] In addition, increased pro-inflammatory mediators disrupt the blood–brain barrier and provoke neuronal excitability and seizure intensity, thus contributing to neuronal cell loss.[34–36] In more detail, previous studies have reported that treatment with TNFα-neutralizing antibodies results in a reduction of neuronal injury in the model of kainic acid-induced epilepsy[35] and that TNFα promotes neuronal excitability by inducing microglial glutamate release and endocytosis of $GABA_A$ receptors.[37,38] Research also indicates that an activated IL-1β system following pilocarpine-induced SE is involved in neuronal death and blood–brain barrier (BBB) disruption[34] and that IL-1β promotes *N*-methyl-D-aspartate (NMDA) receptor function by enhancing tyrosine phosphorylation of NR2A/B subunits, subsequently leading to neuronal hyperexcitability and irreversible neuronal injury.[39] Therefore, it is important to reduce brain inflammation under epileptic conditions, which can prevent epileptic neuronal damage and improve neuronal function. In this study, results showed that intraperitoneal administration of eugenol prevents apoptotic neuronal damage following epileptic seizures. Furthermore, eugenol treatment reduced glial activation induced by SE onset and the elevated levels of TNFα and IL-1β in the epileptic hippocampus. Moreover, our results showed that eugenol treatment suppressed the activation of NF-κB pathway, which has been considered a prototypical pro-inflammatory signaling pathway in the expression of several inflammatory cytokines, including TNFα and IL-1β.[40,41] These findings suggest that eugenol plays a neuroprotective role in SE-induced neuronal damage by alleviating reactive gliosis and enhancing the anti-inflammatory capacity.

The neuroinflammatory cascade is a common pathological factor in the development of various neurological diseases, including Alzheimer's disease, Parkinson's disease, and ischemic stroke.[8] The NLRP3 inflammasome is an intracellular multiprotein complex that comprises an NLRP3 sensor, an ASC adaptor containing a caspase-recruitment domain, and a caspase 1 enzyme.[42] In the formation of the inflammasome complex, NF-κB, a key regulator of pro-inflammatory cytokines,[41] induces the transcriptional expression of NLRP3,[43] which in turn promotes a substantial release of inflammatory cytokines.[44] Accumulating evidence suggests that the NLRP3 inflammasome plays a crucial role in the pathophysiology of epileptic seizures. Previous studies have demonstrated that the expression level of NLRP3 is upregulated in the brains of epileptic patients,[45] and inhibition of the NLRP3 inflammasome could provide neuroprotection by reducing the expression of pro-inflammatory cytokines such as IL-1β, IL-18, and TNFα in experimental models of SE.[46–48] Similar to previous evidence, we found a significant increase in the expression of the NF-κB activation-mediated NLRP3 inflammasome complex in the hippocampus of SE-induced mice. However, these effects were inhibited by eugenol treatment. These findings indicate that eugenol can reduce seizure-induced neuroinflammatory processes by inhibiting the NLRP3 inflammasome pathway.

Accumulating studies have shown the beneficial effects of eugenol under various neuropathological conditions. For instance, previous study reported that eugenol treatment reduced the histopathology of traumatic brain injury, such as BBB breakdown and brain edema, and the behavioral consequences of trauma.[49] In addition, eugenol alleviated cerebral ischemia–reperfusion injury by promoting AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR)/p70S6 kinase (P70S6K) pathway-dependent autophagy.[50] This evidence suggests that eugenol may be a beneficial phytoconstituent for preventing neuronal damage in neuropathological conditions. In line with this evidence, our findings demonstrate that post-treatment with eugenol could protect the neuronal damage through suppression of excessive inflammatory responses following epileptic seizures. However, there are some limitations to this study. Since our observation focused on the beneficial effects of eugenol during the subacute phase of epilepsy, the explicit effects of eugenol through a prominent antiepileptogenic process need to be verified in subsequent experiments prior to prospective clinical applications. In addition to our observations in this study, we cannot exclude the possibility that eugenol administration may be involved in other mechanisms for protecting neuronal damages during epileptic progression. Therefore, whether eugenol treatment can prevent the occurrence of epileptogenesis by controlling aberrant cellular and network activity will be elucidated in the near future.

In conclusion, we demonstrated that eugenol prevents neuronal damage caused by epileptic seizures via its anti-inflammatory effects in a mouse model of pilocarpine-induced SE. Our findings provide new evidence that eugenol may be a beneficial phytoconstituent for the prevention of epileptic neuropathology.

AUTHORS' CONTRIBUTIONS

KHJ contributed to the conceptualization. JZ and KHJ designed the experiments. JZ and SP performed the experiments. JZ, SP, and KHJ collected and analyzed the data. KHJ contributed to the funding acquisition. JZ and KHJ wrote the manuscript. KHJ and W-JK revised the manuscript. CHK and W-JK contributed to the project administration. All authors have consented to the submission of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID ID

Kyoung Hoon Jeong https://orcid.org/0000-0002-6100-9936

SUPPLEMENTAL MATERIAL

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