

Amentoflavone protects hippocampal neurons: anti-inflammatory, antioxidative, and antiapoptotic effects

Zhen Zhang^{1,2,#}, Tao Sun^{1,2,#}, Jian-guo Niu¹, Zhen-quan He¹, Yang Liu², Feng Wang^{1,2,*}

1 Ningxia Key Laboratory of Cerebrocranial Disease, Incubation Base of National Key Laboratory, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region, China

2 Department of Neurosurgery, General Hospital of Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region, China

*Correspondence to:

Feng Wang, Ph.D., nxwwang@163.com.

These authors contributed equally to this work.

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Abstract

Amentoflavone is a natural biflavone compound with many biological properties, including anti-inflammatory, antioxidative, and neuroprotective effects. We presumed that amentoflavone exerts a neuroprotective effect in epilepsy models. Prior to model establishment, mice were intragastrically administered 25 mg/kg amentoflavone for 3 consecutive days. Amentoflavone effectively prevented pilocarpine-induced epilepsy in a mouse kindling model, suppressed nuclear factor- κ B activation and expression, inhibited excessive discharge of hippocampal neurons resulting in a reduction in epileptic seizures, shortened attack time, and diminished loss and apoptosis of hippocampal neurons. Results suggested that amentoflavone protected hippocampal neurons in epilepsy mice *via* anti-inflammation, antioxidation, and antiapoptosis, and then effectively prevented the occurrence of seizures.

Key Words: nerve regeneration; brain injury; epilepsy; neuroprotection; apoptosis; nuclear factor- κ B; brain inflammation; interleukin-6; interleukin-1 beta; inducible nitric oxide synthase; nitric oxide; prostaglandin E2; NSFC grant; neural regeneration

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Introduction

Epilepsy, one of the most common neurological disorders, is characterized by recurrent, usually unprovoked, epileptic seizures, and is followed by cognitive, psychosocial, and social consequences (Chang et al., 2003). Therapeutic strategies available to treat epilepsy have only been able to treat the symptoms and have often proven ineffective. The most significant challenge is to prevent epileptogenesis, not merely to relieve its symptoms. At present, there is no antiepileptic drug that performs this function. Thus, there is an urgent need to develop novel therapeutic interventions or disease-modifying therapies. With new treatment strategies, we can completely or partially prevent the emergence of spontaneous recurrent seizures.

Epileptogenesis is a process where various insults induce the brain to generate a cascade event, thereby enhancing susceptibility of seizures and generating spontaneous recurrent seizures (Loscher et al., 2010). In the last decade, clinical and experimental evidence has shown that brain inflammation is a common substrate in epilepsies of different etiologies (Deurwaerdere et al., 2012; Kobow et al., 2012). Experimental studies have shown that once seizures develop, they contribute to the perpetuation of inflammation in the brain *via* mechanisms that may involve transcription of inflammatory genes

or post-translational changes in cytokine release machinery (Vezzani et al., 2013). Therefore, the use of anti-inflammatory drugs in epilepsy is a promising therapeutic strategy. In recent years, research on medicinal plants has become a hot topic. Amentoflavone, a polyphenolic compound derived from extracts of *Selaginella tamariscina*, has been found to possess anti-inflammatory (Banerjee et al., 2002; Woo et al., 2005; Huang et al., 2012; Tsai et al., 2012), antioxidative (Yamaguchi et al., 2005; Xu et al., 2009; Erdogan-Orhan et al., 2011), anti-viral (Ma et al., 2001; Lee et al., 2011; Wilsky et al., 2012; Coulerie et al., 2013), anti-tumor (Lee et al., 2011; Siveen and Kuttan, 2011; Tarallo et al., 2011), and anti-radiation (Park et al., 2011; Lee et al., 2012) effects, as well as a neuroprotective effect (Kang et al., 2005; Shin et al., 2006; Sasaki et al., 2010; Thapa et al., 2011; Ishola et al., 2012, 2013). Amentoflavone offers inhibits nuclear factor- κ B (NF- κ B)-mediated inducible nitric oxide synthase, which generates nitric oxide (NO) (Woo et al., 2005). Therefore, amentoflavone prevents the inflammatory response induced by NO. NO is able to cause a cascade effect and a series of adverse reactions including tissue damage and apoptosis (Meffert et al., 2005). Amentoflavone exerts a regulatory effect on the oxidant/antioxidant balance by inhibiting production of inflammatory mediators, including inducible

nitric oxide synthase, cyclooxygenase-2, pro-inflammatory cytokines, such as tumor necrosis factor- α , interleukin (IL)-1 β and IL-6, and NF- κ B signal transduction pathways in cell culture or *in vivo* (Guruvayoorappan et al., 2008; Ishola et al., 2013; Sakthivel et al., 2013).

Based on these findings, it is reasonable to hypothesize that amentoflavone may play a neuroprotective role in epilepsy *via* anti-inflammatory effects.

Materials and Methods

Animals

Kunming mice (male, 28–32 g, 5–6 weeks old) were obtained from the Laboratory Animal Center of Ningxia Medical University in China (license No. SCXK (Ningxia) 2011-0001). The animals were assigned to groups of six mice per cage in a controlled environment at room temperature and humidity of 60–65% with a 12-hour light and dark cycle (08:00–20:00) in a pathogen-free colony. Food, in the form of dry fodder, and water were freely given *ad libitum*. This study was approved by the Animal Ethics Committee of Ningxi Medical University, China. All efforts were made during the experiment to use a minimum number of animals and to minimize animal suffering. Mice were maintained in the colony for 1–2 weeks prior to experimental procedures.

Establishment of mouse kindling models and drug administration

All drugs were freshly prepared before used. All drugs for intragastric administration were dissolved and suspended in gum acacia solution (1%).

A total of 150 mice were divided equally into five groups: amentoflavone pre-treated group, amentoflavone-treated group, valproate-treated group, non-treated epilepsy group, and blank control group. The amentoflavone pre-treated group ($n = 30$) was intragastrically administered 25 mg/kg amentoflavone (Sigma-Aldrich, St Louis, MO, USA) for 3 consecutive days, once a day, prior to pilocarpine injection. The non-treated epilepsy group ($n = 30$) and the blank control group ($n = 30$) were simultaneously given the same amount of 0.9% saline. The amentoflavone-treated group ($n = 30$) and valproate-treated group ($n = 30$) were intragastrically given amentoflavone (25 mg/kg) and sodium valproate (20 mg/kg; Sanofi-Aventis Pharmaceutical Co., Hangzhou, China) respectively at 1 hour after status epilepticus. In each group, a third of the animals were used for paraffin embedding, a third were used for western blot analysis, and the remaining third were used for prostaglandin E2 and cytokine assays.

To establish the kindling model, n-methylscopolamine bromide (1 mg/kg; Laien Pharmaceutical Co., Xuzhou, China) was injected subcutaneously. After 30 minutes, the appropriate dose of pilocarpine (300 mg/kg; Sigma-Aldrich) was intraperitoneally injected at 2 hours after status epilepticus. Seizures were terminated with chloral hydrate. Behavioral observations were performed, and seizures were scored according to the Racine's scale. Partial seizures were defined

as stages 1–2, while secondarily generalized seizures were defined as stages 3–5.

Electroencephalogram (EEG) measurement

The EEG acquisition electrodes, with a diameter of 0.15 mm, consisted of NiCr enameled wire and miniature electronic connectors. The ends of the enameled wire and miniature electronic connectors were welded together. To reduce interference signal, the element jacks were electrically insulated with 704 silicone rubber, and the other end of the enameled wire (2 mm) was exposed. Mice were fixed on a brain stereotaxic instrument after anesthesia with sodium pentobarbital (80 mg/kg, intraperitoneally), and then the skull was fully exposed. Bilateral hippocampus was positioned 2.3 mm posterior to bregma, 2.1 mm lateral to the sagittal suture, and 2.0 mm subdural. A 0.2-mm diameter hole was drilled at this point. Sterilized electrodes were inserted into the hippocampus and fixed on the skull surface with dental cement. EEG was conducted at 1 week after surgery.

Alpha-Lab 4-channel signal acquisition and processing workstation (Alpha Omega Ltd., Nazareth, Israel) was used to record electrical activity in mice. The stable signal was selected without significant interference for analysis. Mice were allowed to freely move during the acquisition process. Signals were stored in a computer, and following amplification, they were filtered and digitally transformed, and analyzed using NeuroExplorer software (Alpha Omega Ltd.).

Collection of hippocampus tissue samples

At 72 hours after pilocarpine injection, mice were intraperitoneally anesthetized with 80 mg/kg sodium pentobarbital and were intracardially perfused on an ice tray with 40 mL saline that contained 100 U/mL heparin. Mice were then perfused with 4% paraformaldehyde (soluble in 0.01 M phosphate-buffered saline, pH 7.4) in the same manner. The brains were then rapidly removed and placed in 4% paraformaldehyde for 20 hours. Samples were trimmed with a blade, and then placed in fixatives for approximately 24 hours at room temperature. Samples (one sample per case) were rinsed under running water for 6 hours, and embedded in paraffin after graded ethanol dehydration. Coronal serial (5 μ m-thick) sections were cut at the level of the dorsal hippocampus and mounted on polylysine-treated slides for Nissl staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and immunohistochemical analysis.

For cytokine and western blot assays, mice were anesthetized in the same manner and intracardially perfused with 40 mL warm (37°C) saline containing heparin (100 U/mL). Tissues were collected from the bilateral hippocampal subfield on a piece of gauze moistened with 0.9% ice-cold saline. Hippocampal samples were stored at –80°C until biochemical analyses. Concentration of total proteins extracted from tissue samples was determined using the bicinchoninic acid assay (Keygen Biotechnology Co., Nanjing, China). The nuclear proteins of the hippocampal samples were extracted using a commercial kit (Keygen Biotechnology Co.).

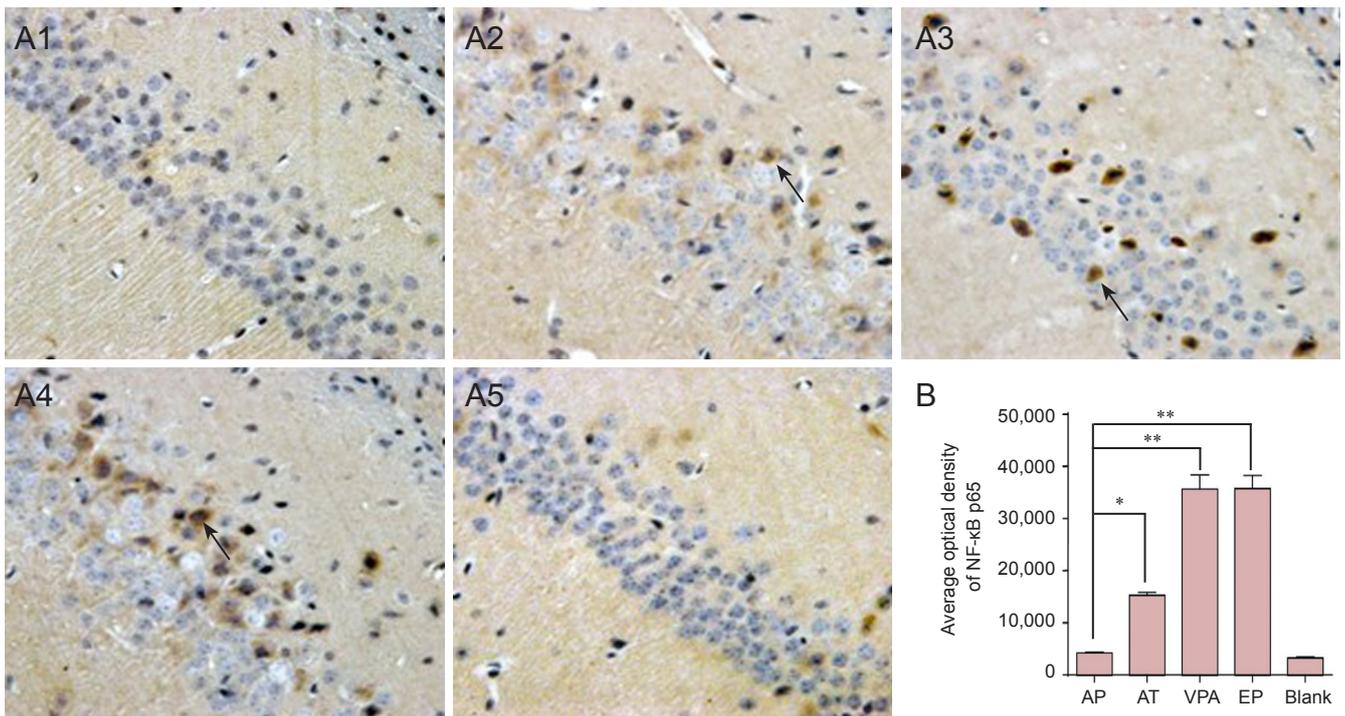


Figure 1 Amentoflavone effects on NF-κB p65 immunoreactivity in the hippocampus of mice with status epilepticus (immunohistochemical staining).

(A) Brain sections were immunostained with antibodies against the p65 subunit of NF-κB (×400). (A1–A5) AP, AT, VPA, EP, and blank groups, respectively. (A1, A5) Occasionally scattered lightly stained NF-κB p65-positive cells. (A2–A4) Many immunoreactive darkly stained positive cells (arrows). (B) Optical density of NF-κB p65-positive cells in hippocampal CA1 substructures of mice sacrificed at 72 hours after status epilepticus (**P* < 0.05, ***P* < 0.01). The data are expressed as the mean ± SEM (*n* = 10) and analyzed using one-way analysis of variance and Dunnett's *post-hoc* test. AP: Amentoflavone pre-treated group; AT: amentoflavone-treated group; VPA: valproate-treated group; EP: non-treated-epilepsy group; Blank: blank control group; NF: nuclear factor.

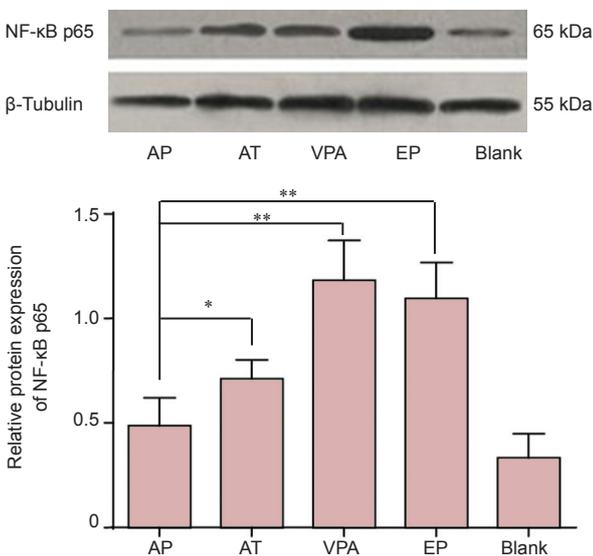


Figure 2 Effects of amentoflavone on NF-κB p65 protein expression in the hippocampus of mice with status epilepticus (western blot assay).

Quantitative results are expressed as gray value ratio (NF-κB p65/β-tubulin) in all groups. Comparison of the intensity ratio shows significantly decreased NF-κB p65 protein expression in the hippocampus of epileptic mice compared to the AP group (**P* < 0.05, ***P* < 0.01). Data are expressed as the mean ± SEM (*n* = 10) and were analyzed using one-way analysis of variance and Dunnett's *post-hoc* test. AP: Amentoflavone pre-treated group; AT: amentoflavone-treated group; VPA: valproate-treated group; EP: non-treated-epilepsy group; Blank: blank control group; NF: nuclear factor.

Nissl staining

Paraffin sections were de-waxed and rehydrated through a series of washes: xylol for 2 × 10 minutes, 100% ethanol for 2 × 5 minutes, 95% ethanol for 1 × 5 minutes, 90% ethanol for 1 × 5 minutes, 80% ethanol for 1 × 3 minutes, 70% ethanol for 1 × 3 minutes, and finally in distilled water for 5 minutes. Sections were stained in 0.1% cresyl violet solution (dissolved in 0.01% glacial acetic acid) at 37°C for 10 minutes, rinsed quickly in distilled water, differentiated in 95% ethyl alcohol for 30 seconds, and checked microscopically (DP71 CCD microscope system, Olympus, Tokyo, Japan). Sections were dehydrated in 100% alcohol 2 × 5 minutes, de-watered through graded ethanol, and permeabilized with xylol until the cover slips were mounted using neutral resin for permanent preservation. Surviving neurons were quantified at 100× magnification. Three fields of the CA1 region were randomly selected in each slice. The number of intact cells was counted, and the average number of cells was calculated for analysis.

Immunohistochemical analysis

Paraffin sections were de-waxed and rehydrated in the same manner as the Nissl staining. Antigens were unmasked in a bath of citrate buffer using a high-pressure steam station for 8 minutes and cooled to room temperature. After washing in PBS, sections were incubated with Triton X-100 (0.3% in 1× PBS) at room temperature for 10 minutes, washed twice

in 1× PBS, and incubated with 5% bovine serum albumin and 5% normal goat serum for 15 minutes. Slices were then incubated with mouse anti-human NF-κB p65 monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a wet chamber at 4°C overnight. On the next day, sections were placed at room temperature for 30 minutes, washed in PBS for 5 minutes, and incubated with biotin-labeled goat anti-mouse IgG secondary antibodies in working solution at room temperature in a wet chamber for 30 minutes. After 3,3'-diaminobenzidine staining (R&D Systems Co., Minneapolis, MN, USA), sections were washed in PBS, counterstained with hematoxylin for 30 seconds, and washed again with running water for 10 minutes. Images were captured using the DP71 microscope-camera system (Olympus). Cell counting was performed in the CA1 subfield of the dorsal hippocampus according to the Paxinos and Franklin mouse atlas (Paxinos and Franklin, 2001). The total number of positive cells was quantified in 1,280 × 1,024 frames using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The amount of NF-κB p65 immunoreactive cells was quantified by the Gel-Pro analyzer software (Media Cybernetics, Inc., Rockville, MD, USA) and expressed as the average optical density calculated by Gel-Pro software.

TUNEL staining

Paraffin sections were de-waxed and rehydrated as described above. Slides were incubated for 15 minutes at room temperature in proteinase K working solution (20 μg/mL in 10 mM Tris/HCl, pH 7.4–8.0; Roche Diagnostics, Mannheim, Germany). The following steps were conducted in accordance with the manufacturer's instructions for the In Situ Cell Death Detection Kit (Roche Diagnostics). A TUNEL-negative control was obtained by omitting TdT from the labeling mix, and TUNEL specificity was verified by comparing labeling with cellular morphology (Olympus DP71 CCD microscope system, Olympus). The steps of counterstaining and cover slip mounting in TUNEL staining are identical to the steps of immunohistochemistry. TUNEL-positive cells were quantified at 400× magnification.

Nitrite measurement, prostaglandin E2, and cytokine assays

The bilateral hippocampus was put into a diethyl pyrocarbonate-treated Eppendorf tube. A total of 1 mL PBS (pH 7.0–7.4) containing 1 μg/L protease inhibitors was added on ice for 10 minutes after mixing and grinding. Samples were centrifuged at 2,000–3,000 × g for 20 minutes. The supernatants were collected, and prostaglandin E2 production was determined using an ELISA kit (Shanghai Xitang Biotechnology Co., Shanghai, China) according to the manufacturer's instructions. NO production was assayed using Griess reagent system (Promega, Madison, WI, USA). Briefly, the samples were mixed with an equal volume of Griess reagent and incubated at room temperature for 15 minutes. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined using a sodium nitrite

serial dilution standard curve.

Tissue-free supernatants were collected and stored at –2°C until they were assayed for cytokines. IL-1β and IL-6 concentrations in the tissue supernatants were measured using an ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions.

Western blot assay

Western blot assay for NF-κB p65 subunit was performed on proteins extracted from nuclear fractions of hippocampal samples. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The primary antisera included a mouse anti-human NF-κB p65 monoclonal antibody (1:500, 4°C, 12 hours; Santa Cruz Biotechnology), or β-tubulin (1:5,000, 4°C, 12 hours). The secondary antibodies in working solution contained biotin-labeled goat anti-mouse IgG secondary antibodies (Invitrogen Corporation, Carlsbad, CA, USA) for NF-κB p65 (1:5,000, 25°C, 2 hours) and β-tubulin (1:10,000, 25°C, 2 hours). Specific antibody-antigen complexes were detected using an enhanced chemiluminescence western blot detection system (Cell Signaling Technology, Danvers, MA, USA). The amount of protein was quantified by the Gel-Pro analyzer software (Media Cybernetics), and was expressed as the gray value ratio of target protein to β-tubulin protein.

Statistical analysis

All data obtained from measurements were expressed as the mean ± SEM. Statistical comparisons between two groups or among multiple groups were performed by one-way analysis of variance with Dunnett's *post-hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of amentoflavone on NF-κB p65 immunoreactivity and protein expression in the mouse hippocampus following experimental status epilepticus

The first series of experiments established NF-κB p65 activation in hippocampal CA1 neurons following experimental status epilepticus. In the blank control group, NF-κB p65-positive neurons were rarely detected in the hippocampus in immunohistochemical experiments (**Figure 1**). Following status epilepticus, positive signals were detected in CA1 subfield cells within the amentoflavone-treated group, valproate treated-group, and non-treated epilepsy group. However, NF-κB p65 protein was weakly detected in the amentoflavone pre-treated and blank control groups. Following status epilepticus, NF-κB p65 protein was detected in the nuclei and cytoplasm of CA1 subfield cells. Western blot assay revealed significantly increased NF-κB p65 expression in the ipsilateral hippocampus following status epilepticus (**Figure 2**). These results suggested that amentoflavone inhibited activation and nuclear translocation of NF-κB subunits p65. Thus, amentoflavone administration could suppress hippocampal inflammation by suppressing NF-κB signal transduction pathways.

Effects of amentoflavone on neurons in hippocampal CA1 of mice with status epilepticus

Histological analysis demonstrated that amentoflavone decreased neuronal damage in the hippocampal CA1 subfield after status epilepticus (Figure 3). In the blank control and amentoflavone pre-treated groups, CA1 neurons were almost intact at 72 hours after status epilepticus. In contrast, neurons were severely degenerated in the amentoflavone-treated group ($P < 0.05$), valproate-treated group ($P < 0.05$), and non-treated epilepsy group ($P < 0.01$). In the amentoflavone-treated group, neurons were fairly well preserved and sparse damage was observed in the CA1 subfield. Most of the morphologically damaged neurons stained with cresyl violet were also positive for TUNEL (Figure 4). Sparse TUNEL-positive cells were observed in the CA1 subfield in the amentoflavone pre-treated group ($P < 0.05$). No significant difference was observed between the blank control and amentoflavone pre-treated groups.

Effects of amentoflavone on activated EEG signals during experimental status epilepticus

These series of experiments examined whether NF- κ B signaling affected sustained seizure activity during experimental status epilepticus (Figure 5).

Compared to the blank control group, the local field potential signal in the amentoflavone-treated group and non-treated epilepsy group appeared unstable after injection of pilocarpine, with continuous epileptiform discharges and accompanied seizures of grade III–V. After model establishment, no grand mal seizures occurred, but grade I–II seizures occasionally occurred, and the attack time was significantly shortened in the amentoflavone pre-treated group and valproate-treated group. Manifestations of local field potential signal were similar to the blank control group, and were stable compared with the non-treated epilepsy group.

Effects of amentoflavone on NO and prostaglandin E2 production in the hippocampus of mice with status epilepticus

NO and prostaglandin E2 levels in the supernatants of hippocampal tissue samples were determined with the Griess assay and ELISA kit. Pre-treatment with amentoflavone in the amentoflavone pre-treated group resulted in significantly decreased NO and prostaglandin E2 production compared to the non-treated epilepsy group ($P < 0.01$) (Figure 6A, B).

Effects of amentoflavone on IL-1 β and IL-6 production in the hippocampus of mice with status epilepticus

Pre-treatment with amentoflavone resulted in significantly decreased IL-6 and IL-1 β production in the hippocampus of mice in the amentoflavone pre-treated group compared to the non-treated epilepsy group ($P < 0.01$) (Figure 6C, D).

Discussion

A widely accepted hypothesis holds that there is a latent period between brain insults and the onset of symptomatic epi-

lepsy, which could last for months to years. This latent period characterizes many cases of symptomatic epilepsy, including a cascade of poorly understood changes that transform the non-epileptic brain into one that generates spontaneous recurrent seizures (Loscher et al., 2010). This insult-induced process is called epileptogenesis. Recently, the activation of specific pro-inflammatory pathways has been demonstrated in human and experimental epileptic brain tissue during the epileptogenic process (Vezzani et al., 2013). Clinical and neuropathological evidence has shown that inflammation could play a central role in seizure disorders without infectious or immune-mediated etiology. Several clinical studies demonstrated increased levels of inflammatory mediators, such as IL-6, tumor necrosis factor alpha, and IL-1 β , in serum or cerebrospinal fluid. The activation of the cytokine network in patients with refractory epilepsy may vary not only on seizure severity or duration, but also on epilepsy syndromes. In short, inflammation is induced by recurrent seizures, and in turn, epilepsy could increase the inflammation (De Simoni et al., 2000; Dhote et al., 2007; Ravizza et al., 2008, 2011). In kainate-induced seizures models, NF- κ B activity rapidly increases in hippocampal neurons within 4–16 hours following kainate-induced seizures, and is followed by a delayed and sustained increase in glial cells (Matsuoka et al., 1999). Hippocampal sclerosis specimens of temporal lobe epilepsy surgery show overexpression of NF- κ B p65 in glial cells and pyramidal cells (Crespel et al., 2002). Other studies show that blockade of IL-1 β signaling in the brain using IL-1 receptor antibody drastically reduces seizures in various animal models (De Simoni et al., 2000; Vezzani et al., 2000). Overexpressing the soluble form of human IL-1 receptor antibody in astrocytes results in an intrinsic resistance to seizures (Vezzani et al., 2000). This evidence indicates that brain inflammation contributes to seizures, rather than being considered a mere epiphenomenon of the pathology.

Amentoflavone is widespread in Selaginella in the natural world. Scientists have been conducting extensive studies on its anti-inflammatory (Banerjee et al., 2002; Woo et al., 2005; Huang et al., 2012; Tsai et al., 2012), anti-tumor (Lee et al., 2011; Siveen and Kuttan, 2011; Tarallo et al., 2011), and neural protective mechanisms (Kang et al., 2005; Shin et al., 2006; Sasaki et al., 2010; Thapa et al., 2011; Ishola et al., 2012, 2013) in recent years, but no study has reported on its effect on epilepsy. Studies have confirmed that amentoflavone can inhibit the ability of NF- κ B-mediated inducible nitric oxide synthase, which generates NO, to reduce the production of NO, thereby preventing the inflammatory response (Woo et al., 2005). As an important mediator of inflammation, NO induces a series of cascade effects and causes a variety of adverse reactions including tissue damage and apoptosis (Meffert et al., 2005). Studies have shown that there is a variety of neuronal apoptosis in animal models of epilepsy (Henshall et al., 2005). Therefore, we hypothesized that the neural protective effect provided by amentoflavone takes place by inhibiting NF- κ B activity and thereby regulating inflammation and apoptosis during

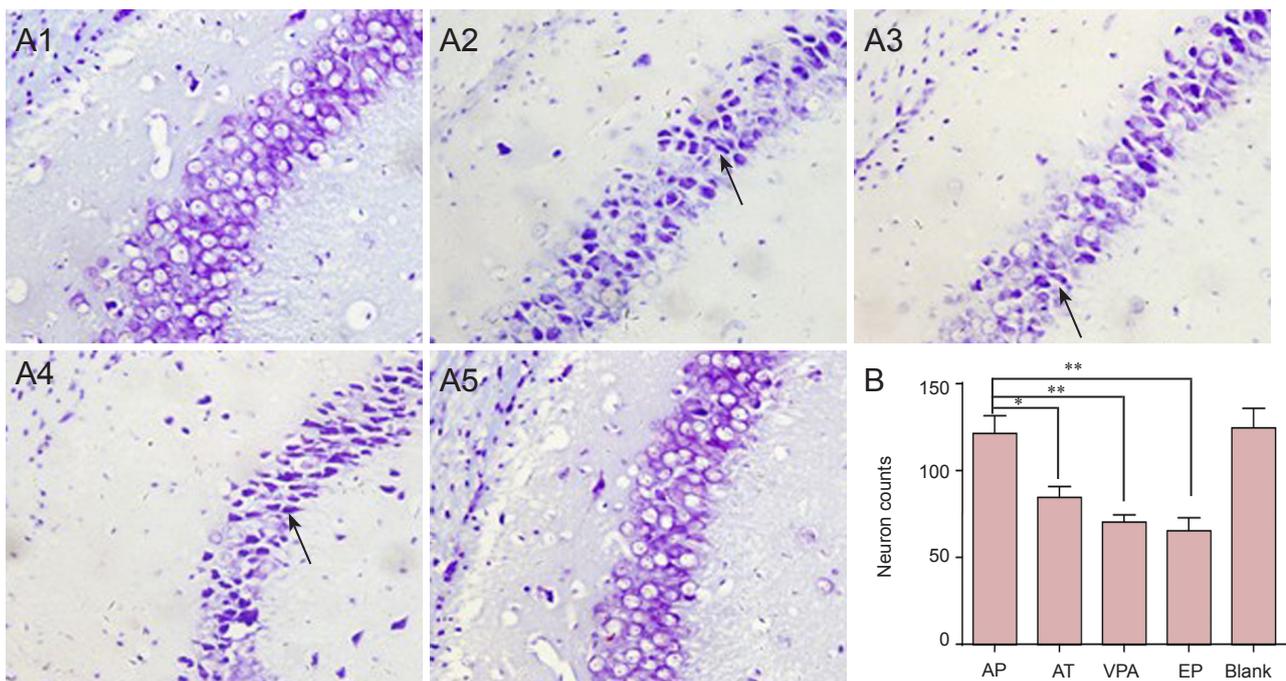


Figure 3 Amentoflavone effects on neuronal injury in the hippocampal CA1 of mice with status epilepticus (Nissl staining). (A) Nissl staining was used to identify Nissl bodies and the extent of neuronal damage in the CA1 subfield ($\times 400$). (A1–A5) AP, AT, VPA, EP, and blank groups, respectively. (A2–A4) Hippocampal CA1 neurons were loose and absent; the cytoplasm was stained in different shades; nucleoli were missing or indistinct (arrows). (A1, A5) Neurons were neatly arranged and displayed sharp edges. Nissl bodies showed visible cytoplasm. (B) Neuronal counts. In the amentoflavone-treated group, neurons were fairly well preserved and sparse in the CA1 subregion. $*P < 0.05$, $**P < 0.01$. Data are expressed as the mean \pm SEM ($n = 10$) and analyzed using one-way analysis of variance and Dunnett's *post-hoc* test. AP: Amentoflavone pre-treated group; AT: amentoflavone-treated group; VPA: valproate-treated group; EP: non-treated-epilepsy group; Blank: blank control group.

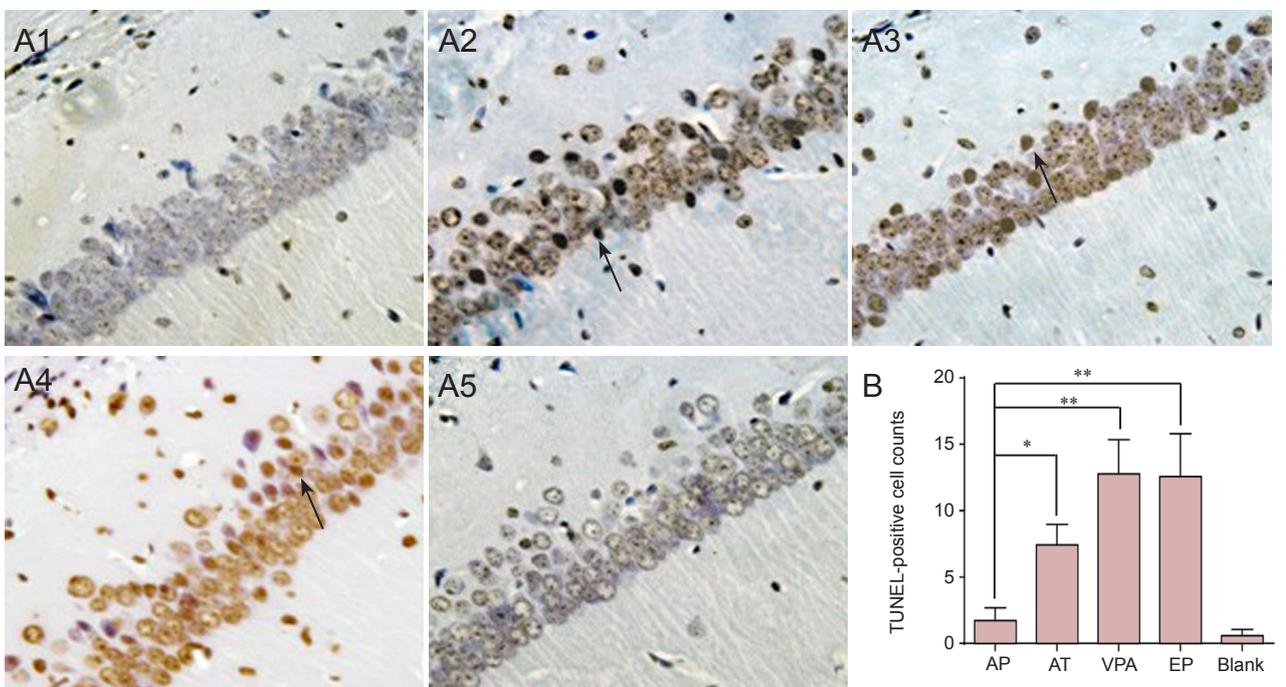


Figure 4 Amentoflavone effects on cell apoptosis in the hippocampal CA1 of mice with status epilepticus (TUNEL staining). (A) TUNEL staining was used to observe differences in apoptosis ($\times 400$). (A1–A5) AP, AT, VPA, EP, and blank groups. TUNEL-positive cells were quantified at $400\times$ magnification. (A1) Sparse TUNEL-positive cells in CA1 subfield; (A1, A5) scarce TUNEL-positive cells; (A2–A4) significantly increased number of TUNEL-positive cells (arrows). (B) TUNEL-positive cell counts. No significant difference was observed between the blank and AP groups. $*P < 0.05$, $**P < 0.01$. Data are expressed as the mean \pm SEM ($n = 10$) and analyzed using one-way analysis of variance and Dunnett's *post-hoc* test. AP: Amentoflavone pre-treated group; AT: amentoflavone-treated group; VPA: valproate-treated group; EP: non-treated-epilepsy group; Blank: blank control group; TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling.

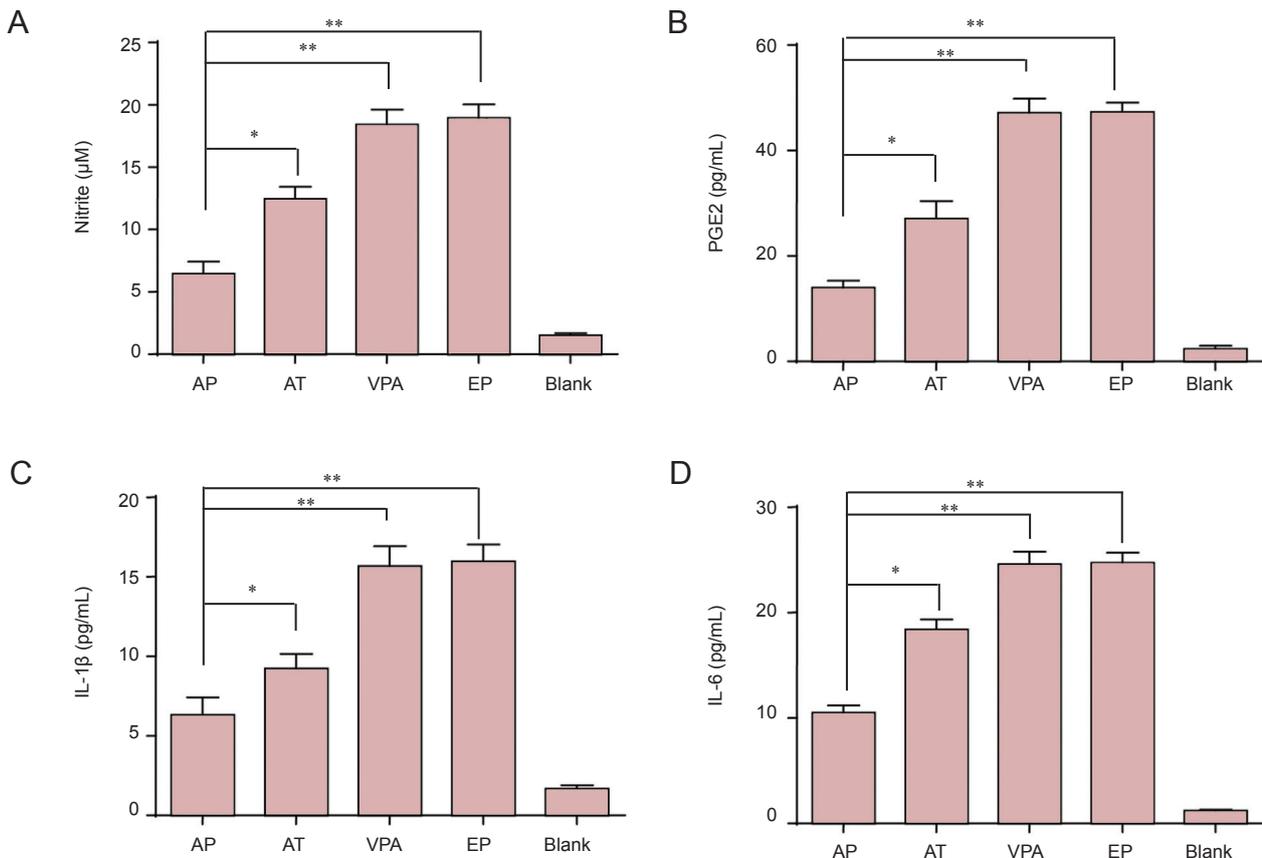


Figure 6 Effects of amentoflavone on NO, PGE2, IL-1β, and IL-6 level in the hippocampus of mice with status epilepticus. (A, B) Concentrations of NO (Griess reagent system) and PGE2 (ELISA) were analyzed in samples using one-way analysis of variance to determine statistical significance. Pre-treatment with amentoflavone resulted in significantly decreased NO and PGE2 production in the AP group compared to other control groups (* $P < 0.05$, ** $P < 0.01$). (C, D) Concentrations of IL-1β and IL-6 (ELISA) in samples were analyzed using one-way analysis of variance to determine statistical significance. Pre-treatment with amentoflavone resulted in significantly decreased IL-6 and IL-1β production in the AP group compared to other control groups (* $P < 0.05$, ** $P < 0.01$). Data are expressed as the mean \pm SEM ($n = 10$) and were analyzed using one-way analysis of variance and Dunnett's *post-hoc* test. AP: Amentoflavone pre-treated group; AT: amentoflavone-treated group; VPA: valproate-treated group; EP: non-treated-epilepsy group; Blank: blank control group. NO: Nitric oxide; PGE2: prostaglandin E2; IL: interleukin.

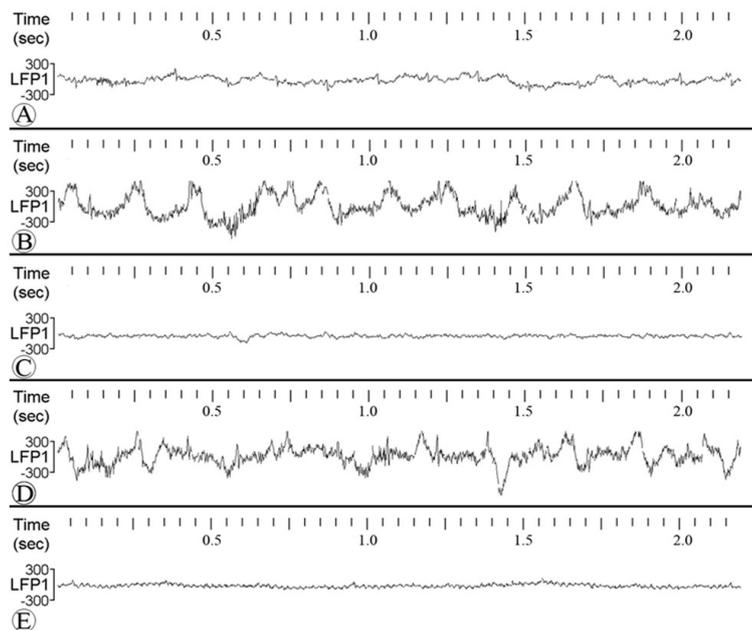


Figure 5 Effect of amentoflavone on activated EEG signals in mice with status epilepticus. Behavioral manifestations and EEG: EEG acquisition and analysis using LFP test methods. After injection of pilocarpine for 5–10 minutes, mice in the non-treated epilepsy group (D) suffered from limbic system disorders, including loss of movement, salivation, shaking whiskers, wet-dog shakes, facial twitching, and balance disorders. At 12–30 minutes, the mice exhibited forelimb clonic and/or tonic-clonic upright or falling, and presented with status epilepticus grade III–V seizures by 20–40 minutes. The blank control group (E) had no seizures. LFP signal in amentoflavone-treated group (B) was similar to the non-treated epilepsy group (D). After pilocarpine injection, the LFP signal in the non-treated epilepsy group appeared instable, with continuous epileptiform discharges accompanied by seizures. After model establishment, there were no grand mal seizures, but the mice experienced occasional grade I–II attacks, and attack time was significantly shortened in the amentoflavone pre-treated group (A) and valproate-treated group (C). Manifestations of LFP signal were similar to the blank control group, and stable compared with the non-treated epilepsy group. EEG: Electroencephalogram; LFP: local field potential; sec: second.

epilepsy in hippocampal neurons.

NF- κ B, which forms a DNA-binding dimer, comprises p65 and p50 and plays an important role in various inflammatory processes, including inducible nitric oxide synthase induction. The NF- κ B dimer is located in the cytoplasm and functions as an inactive complex bound to I- κ B α , which is phosphorylated and subsequently degraded, and then dissociates to produce activated NF- κ B (Guerrini et al., 1995; Meberg et al., 1996). As in the nervous system, functional NF- κ B complexes are present in all brain cell types, including neurons, astrocytes, microglia, and oligodendrocytes (O'Neill et al., 1997). In neurons and glial cells, receptor-linked signal transduction pathways that eventually result in NF- κ B activation, such as those activated by tumor necrosis factor alpha and Fas ligand, have also been reported (Bruce et al., 1996). Previously confirmed experiments show that amentoflavone completely blocks nuclear translocation of p65 and abolishes degradation of I- κ B α in cells treated with lipopolysaccharide. These results indicate that I- κ B kinase-mediated phosphorylation and subsequent degradation of I- κ B α is a pharmacological target of amentoflavone (Karin et al., 2000). The I- κ B kinase complex can be activated by a variety of upstream kinases, such as protein kinase C and the tyrosine kinase family (Huang et al., 2003; Trushin et al., 2003). Thus, amentoflavone may also act on these upstream kinases. A protective role for seizure-induced neuronal NF- κ B activation has been suggested by studies showing that intraventricular infusion of κ B decoy DNA prior to administration of kainate results in a significant increase in neuronal death in hippocampal subregions CA1 and CA3 (Yu et al., 1999).

The present study confirms that amentoflavone inhibits activation and nuclear translocation of NF- κ B subunits p65. These results suggest that amentoflavone administration effectively overwhelms inflammation in the hippocampus by inhibiting NF- κ B signal transduction pathways. Thus, blockade of NF- κ B signal transduction pathways may be one of the major mechanisms underlying the prevention of pilocarpine-induced epilepsy. Our results are in agreement with previous studies that show that flavonoids and their metabolites may interact with mitogen-activated protein kinase signaling pathways. It is well known that mitogen-activated protein kinase signaling pathways play an important role in regulating diverse cellular functions, including NF- κ B expression (Kuo et al., 2011; Lee et al., 2012). After being activated upstream, p38-mitogen-activated protein kinases allow activation and nuclear translocation of NF- κ B. Thereby, amentoflavone may interact with proteins involved in the mitogen-activated protein kinase pathway by a direct inhibitory effect and represent a specific mechanism by which amentoflavone may avert epilepsy.

In summary, amentoflavone administration exerts preventive effects against seizures and neuroprotective effect in pilocarpine-induced epilepsy in mice, which is mediated by changes in the inflammation/anti-inflammatory balance of the hippocampus, as well as reduces seizures, decreases damage and apoptosis within hippocampal neurons, inhibits

production of inflammatory mediators, including NO, prostaglandin E2, and pro-inflammatory cytokines such as IL-1 β and IL-6, and inhibits NF- κ B signal transduction pathways. Amentoflavone has a protective effect on hippocampal neuronal damage caused by the experimental epilepsy.

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