Eupatilin inhibits microglia activation and attenuates brain injury in intracerebral hemorrhage

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Abstract. Inflammation serves a critical role in the pathophysiology of intracerebral hemorrhage (ICH)-induced brain injury. Eupatilin, a pharmacologically active flavone derived from *Artemisia sp.*, has been reported to have antioxidant, anti-inflammatory, anti-allergic and antitumor activities. However, the effect of eupatilin in ICH has not been well studied. The aim of the present study was to investigate the effect of eupatilin on ICH-induced microglial inflammation. The MTT and Transwell migration assay results revealed that eupatilin significantly inhibited microglial migration. It also decreased the production of inflammatory cytokines in erythrocyte lysis-induced BV2 cells, as well as the level of intracellular reactive oxygen species. The anti-inflammatory mechanism of eupatilin was also investigated using ELISAs and western blotting and the results demonstrated that eupatilin was able to inhibit erythrocyte lysis-induced NF-κB activation in BV2 cells. Taken together, the results of the present study suggest that eupatilin serves neurological protective effects via inhibiting microglial inflammation, providing an experimental basis for the use of eupatilin as a therapeutic target for ICH.

Introduction

Intracerebral hemorrhage (ICH) accounts for 10-15% of all strokes and is associated with high mortality and morbidity (1). ICH is characterized by the rupture of cerebral blood vessels and subsequent leakage of blood, including blood-intrinsic factors, into the brain parenchyma (2). Currently, no effective treatment options are available for ICH. Previous studies have reported that inflammation is a key factor that contributes to ICH-induced brain injury (2-4). It is thought that resident microglia and astrocytes are the early inflammatory cells in ICH (4). Activated inflammatory cells release a variety of cytokines, chemokines, free radicals and other potentially toxic chemicals (5-7), which further aggravate brain injury. Therefore, suppressing microglial function may be a promising novel strategy for ICH therapy.

Artemisia princeps Pampanini (family Asteraceae) is an herbal medicine widely used in Korea, China and Japan. Eupatilin, a pharmacologically active flavone derived from *Artemisia sp.* has been reported to have antioxidant, anti-inflammatory, anti-allergy and anti-tumor activities (8-11). Kim *et al* (12) reported that pre-treatment with eupatilin decreased the production of interleukin (IL)-8 and prostaglandin E2 induced by *Bacteroides fragilis* in HT-29 intestinal epithelial cells. Eupatilin has been reported to exert neuroprotective activities against ischemia/reperfusion-induced delayed neuronal injury in mice, increasing the number of viable cells and decreasing the number of degenerating neuronal cells in the hippocampal CA1 region (13). However, the effect of eupatilin in ICH has not been well studied. The aim of the present study was to investigate the effect of eupatilin on ICH-induced microglial inflammation.

Materials and methods

Cell culture and reagents. The murine microglial cell line BV2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. Eupatilin was supplied by Dong-A Pharmaceutical Co. Ltd. (Yong-In, South Korea) and dissolved in dimethylsulfoxide for treatment.

MTT assay. An MTT assay was performed to assess microglia viability. In brief, BV2 cells ($1x10^5$ cells/well) were cultured at 37˚C with various concentrations of eupatilin (1, 10 or 50 μM) for 24 h. Cells were incubated with MTT solution (5 mg/ml) at 37˚C for 4 h, following which dimethylsulfoxide was added and shaken at room temperature for 10 min. The optical density was determined at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transwell migration assay. The migration assay was performed using a Transwell system. The lower compartment

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was filled with 0.5 ml of DMEM containing 1% FBS with 10 μl erythrocyte lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) alone or together with eupatilin (1, 10 or 50 μM). BV2 cells ($1x10^5$ cells/well) were resuspended in 0.1 ml of DMEM and placed in the upper Transwell chamber, which was subsequently incubated for 24 h at 37˚C. Cells on the lower surface of the filter were fixed with 3.7% paraformaldehyde in PBS at 37˚C for 2 h. Cells were then stained with 5% crystal violet for 30 min at 37˚C, washed with PBS three times at room temperature, and the migratory BV2 cells were counted under a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan; magnification, x200) in at least six random fields. All experiments were performed in triplicate.

ELISA. BV2 microglial cells were seeded at a density of $1x10^5$ cells/well in 24-well tissue culture plates and cultured at 37˚C with various concentrations of eupatilin (1, 10 or 50 μM) for 1 h. Subsequently, wells were stimulated with 10 μl erythrocyte lysis buffer and supernatants were removed 3 days later. Tumor necrosis factor-α (TNF-α; cat. no. MTA00B), IL-1β (cat. no. MLB00C) and IL-6 (cat. no. DY406) expression was measured using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance at 450 nm was determined using a microplate reader.

Measurement of intracellular reactive oxygen species (ROS). BV2 cells ($1x10^5$ cells/well) were pre-treated with various concentrations of eupatilin (1, 10 or 50 μM) for 1 h followed by 10 μl erythrocyte lysis buffer stimulation for 24 h. Next, 2-,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 5 μM) was added to the cells at 37˚C for 20 min. Oxidation of the non-fluorescent H_2DCF-DA by intracellular reactive oxygen species (ROS) results in formation of the fluorescent compound 2-,7-dichlorofluorescein (DCF). DCF mean fluorescence intensity (MFI) was monitored with a laser confocal scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Western blotting. BV2 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (5 mM EDTA, 1 mM PMSF, and 1 mM sodium orthovanadate) for 30 min on ice. The protein content was determined using a BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.). Proteins (20 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature, followed by incubation with primary antibodies: Rabbit anti-mouse p-NF-κB p65 (1:1,000; cat. no. sc-135768) and NF-κB p65 (1:1,000, cat. no. sc-71675; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, following which bands were detected using an enhanced chemiluminescent detection kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The relative intensity of protein signals was normalized to the corresponding β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) intensity and was quantified by densitometric analysis using ImageQuant software (version 7.0; GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. All data are presented as the mean ± standard deviation. Each experiment was repeated at least three times in triplicate, unless otherwise stated. Differences between two groups were analyzed using paired Student's t-tests. One-way analysis of variance followed by Student-Newman-Keuls post hoc test was used to compare differences between multiple groups. The results were analyzed using SPSS software (version 20.0; IBM Corp, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of eupatilin on microglia viability. To investigate the effect of eupatilin on microglia viability, cells were treated with various concentrations of eupatilin (1, 10 or 50 μM). As indicated in Fig. 1, eupatilin did not significantly affect microglia viability compared with the control group.

Effects of eupatilin on microglia migration. The effect of eupatilin on microglia migration was assessed using a Transwell assay. As indicated in Fig. 2, erythrocyte lysis stimulation significantly promoted microglia migration compared with the PBS control, whereas eupatilin significantly suppressed erythrocyte lysis-induced microglia migration in a dose-dependent manner.

Effects of eupatilin on inflammation cytokine release. To assess the anti-inflammatory effects of eupatilin in ICH-induced BV2 microglia, cell culture media were collected and TNF-α, IL-1β and IL-6 levels were measured in ICH-induced BV2 cells. As indicated in Fig. 3, erythrocyte lysis stimulation significantly increased the production of TNF-α, IL-1β and IL-6, whereas eupatilin suppressed these increases cytokine in a dose-dependent manner.

Effect of eupatilin on intracellular ROS. It has previously been reported that increased intracellular ROS may serve a critical role in the progression of ICH (5). The effect of eupatilin on intracellular ROS production in BV2 cells was therefore investigated. As indicated in Fig. 4, erythrocyte lysis stimulation significantly increased the production of ROS. However, eupatilin obviously suppressed erythrocyte lysis-induced ROS production in BV2 cells.

Effect of eupatilin on NF-κB activation. It has been reported that NF-κB is an important regulator of cell fate and function in the nervous system (7). The effect of eupatilin on NF-κB activation was investigated using western blotting. As presented in Fig. 5, erythrocyte lysis stimulation significantly induced the expression of p-NF-κB p65, whereas eupatilin reduced erythrocyte lysis-induced NF-κB activation in BV2 cells.

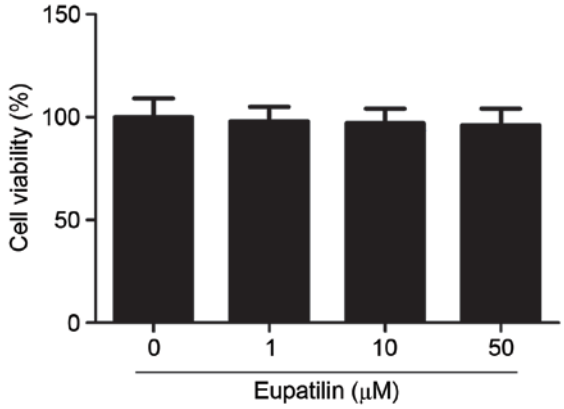

Figure 1. Effects of eupatilin on microglia viability. BV2 microglia ($1x10^5$ cells/well) were treated with 1, 10 or 50 μM eupatilin for 24 h and cell viability was assessed using an MTT assay. Data are presented as the mean ± standard deviation (n=3).

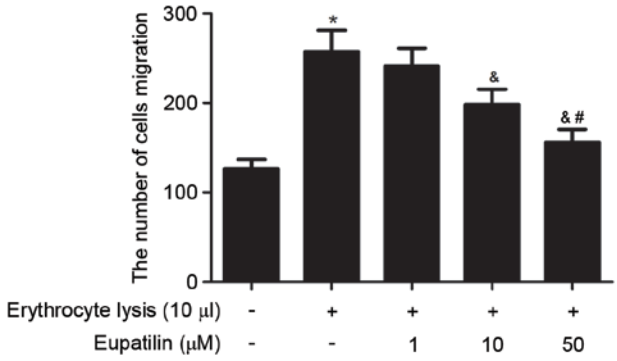

Figure 2. Effects of eupatilin on microglia migration. BV2 microglia ($1x10^5$ cells/well) were treated with 1, 10 or 50 μM eupatilin for 1 h and were subsequently stimulated with 10 μl erythrocyte lysis buffer for 24 h. Microglia migration was detected using a Transwell assay. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control group, &P<0.05 vs. erythrocyte lysis group and #P<0.05 vs. 10 μM eupatilin.

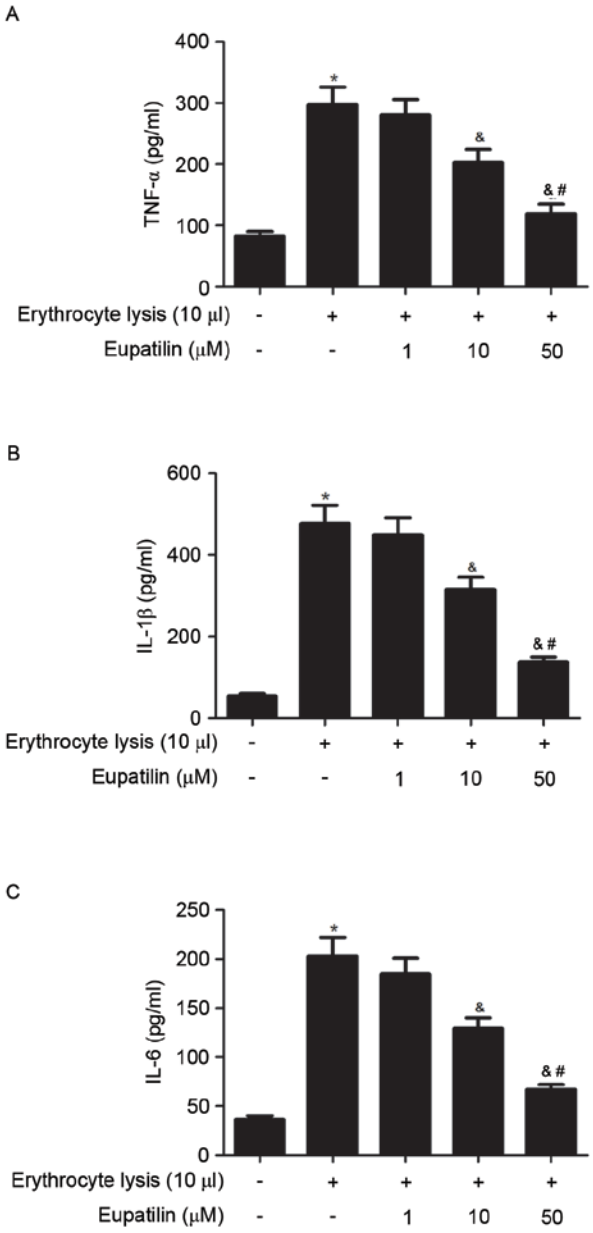

Figure 3. Effects of eupatilin on inflammation cytokine release. BV2 microglia ($1x10^5$ cells/well) were treated with 1, 10 or 50 μM eupatilin for 1 h and were subsequently stimulated with 10 μl erythrocyte lysis buffer for 72 h. Supernatants were collected and ELISA was performed to measure the expression of (A) TNF-α, (B) IL-1β and (C) IL-6. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control group, &P<0.05 vs. erythrocyte lysis group and #P<0.05 vs. 10 μM eupatilin. TNF, tumor necrosis factor; IL, interleukin.

Discussion

Inflammation serves a critical role in the pathophysiology of ICH-induced brain injury; however, the mechanism by which ICH stimulates the inflammatory response remains unclear. In the present study, it was demonstrated that eupatilin significantly inhibited microglial migration. It also decreased the production of inflammatory cytokines and intracellular ROS levels in erythrocyte lysis-induced BV2 cells. The anti-inflammatory mechanism of eupatilin was also assessed and the results revealed that eupatilin was able to inhibit erythrocyte lysis-induced NF-κB activation in BV2 cells.

Microglia are believed to serve a crucial role in the development ICH (13,14). During ICH, microglia migrate into damaged tissue to trigger inflammation and wound healing (14). It has been reported that migration and process motility are typical of activated microglia and serve to induce a number of regulated cellular functions, including cytokine production, phagocytosis and antigen production (14). Furthermore, several studies have reported that microglial migration is increased in erythrocyte lysis stimulated microglia (15,16). In the present study, it was demonstrated that erythrocyte lysis treatment promotes microglial migration, which is consistent with these previous studies. However, eupatilin significantly inhibited microglial migration.

In animal models, microglial cells are activated in the brain following ICH (16). Microglia are critical regulators of the neuron-inflammatory response and major contributors to the excessive production of pro-inflammatory cytokines (2). It has been reported that pro-inflammatory cytokines serve important roles in exacerbating ICH-induced brain injury (17). TNF-α is significantly increased in ICH models and may contribute to the formation of brain edema and brain injury (4,17), while

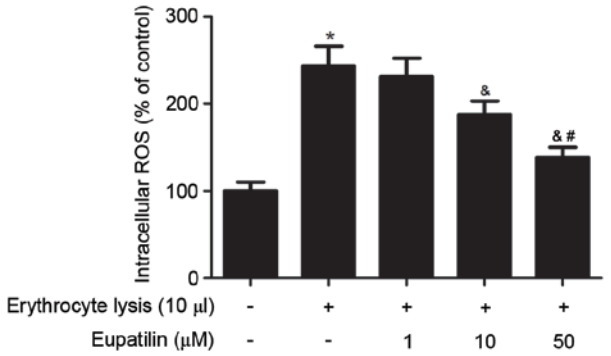

Figure 4. Effect of eupatilin on intracellular ROS. BV2 microglia ($1x10^5$ cells/well) were treated with 1, 10 or 50 μM eupatilin for 1 h and were subsequently stimulated with 10 μl erythrocyte lysis buffer for 24 h. Intracellular ROS was measured using the redox-sensitive fluorescent dye 2-,7-dichlorodihydrofluorescein diacetate. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control group, &P<0.05 vs. erythrocyte lysis group and #P<0.05 vs. 10 μM eupatilin. ROS, reactive oxygen species.

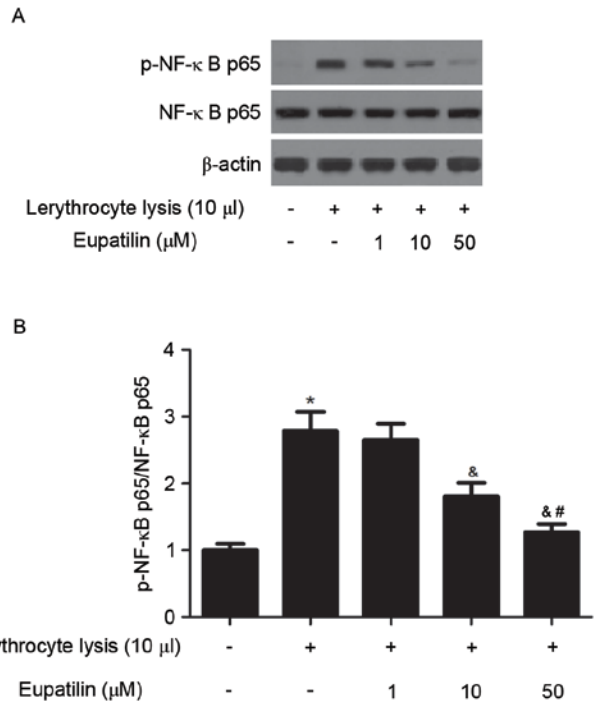

Figure 5. Effect of eupatilin on NF-κB activation. BV2 microglia ($1x10^5$ cells/well) were treated with 1, 10 or 50 μM eupatilin for 1 h and were subsequently stimulated with 10 μl erythrocyte lysis buffer for 24 h. (A) p-NF-κB p65 and NF-κB p65 protein expression was assessed using western blotting. (B) Densitometry was performed to quantify data. Data was also assessed. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control group, &P<0.05 vs. erythrocyte lysis group and #P<0.05 vs. 10 μM eupatilin. NF, nuclear factor; p, phosphorylated.

IL-6 is a multifunctional cytokine that serves an important role in host defense and has major regulatory effects in the inflammatory response (18). The results of the present study demonstrate that erythrocyte lysis stimulation significantly increases the production of TNF-α, IL-1β and IL-6, whereas eupatilin suppresses this effect in a dose-dependent manner. These data suggest that eupatilin may be able to inhibit ICH-induced microglia mediated inflammation.

ROS production following ICH contributes to ICH pathogenesis (19). Several lines of evidence indicate that ROS serve as secondary messengers to encode and enhance the expression of pro-inflammatory factors (20,21). Furthermore, intracellular ROS accumulation in microglia has been reported to trigger the release of inflammatory mediators via the activation of signaling molecules, including mitogen-activated protein kinases and NF-κB (22). In the present study, it was reported that eupatilin downregulates erythrocyte lysis-induced intracellular ROS in BV-2 cells. These results suggest that eupatilin has a neuroprotective effect that is achieved via the inhibition of ROS production in microglia.

NF-κB serves a crucial role in regulating immunity and inflammation in central nervous system injuries, including ICH (23-25). Zhang *et al* (26) reported that NF-κB activation was increased in perihematomal brain tissue following ICH. It has also been suggested that NF-κB activation in microglia following ICH results in the upregulation of pro-inflammatory cytokines, including TNF-α and IL-1β, and contributes to brain injury (27). The present study demonstrates that eupatilin is able to prevent erythrocyte lysis-induced NF-κB activation in BV2 cells. These results suggest that the anti-inflammatory effect of eupatilin may result from inhibition of the NF-κB signaling pathway.

In conclusion, the results of the present study suggest that eupatilin serves a neurological protective effect via inhibiting microglial inflammation. The present study may provide an experimental basis for the use of eupatilin as a therapeutic target for ICH.

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Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to further research being performed, but are available from the corresponding author on reasonable request.

Authors' contributions

HBQ and JL designed the study. LJL, BJN, PL and FX performed the experiments. ZMZ analyzed the data.

Ethics approval and consent to participate

All patients were required to provide written informed consent prior to their inclusion. The study was approved by the Ethical Committee of Dezhou People's Hospital.

Patients' consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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