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Eupatilin attenuates the inflammatory response induced by intracerebral hemorrhage through the TLR4/MyD88 pathway

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A R T I C L E I N F O

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A B S T R A C T

Background: Intracranial hemorrhage (ICH) is one of the most common brain traumas, and inflammation caused by ICH seriously affects the quality of life and prognosis of patients. Eupatilin has been shown to have anti-inflammatory effects in various diseases. However, only one paper has reported that Eupatilin has a therapeutic effect on the inflammatory response caused by ICH and the underlying mechanism needs to be studied.
Methods: We used erythrocyte lysis stimulation (ELS) to induce mouse microglia BV2 as the inflammation model. CCK-8 and Transwell assays were used to detect cell viability and migration. RT-PCR, western blotting, and ELISA were used to detect the secretion of inflammatory factors and the expression of related mechanism proteins. HE staining was used to detect cell edema and death.
Result: We found that ELS significantly increased protein and mRNA levels and secretion of inflammatory factors IL-1β and TNF-α, which Eupatilin attenuated through the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) pathway. The anti-inflammatory effect of Eupatilin was significantly attenuated after siRNA was used to reduce TLR4 expression. The experimental results and mechanism were also verified in TLR4 knockout mice in vivo.
Conclusion: Eupatilin has a therapeutic effect on inflammation caused by ICH. The underlying mechanism may be related to TLR4/MyD88, which brings new hope for clinical patients to improve symptoms and prognosis.

1. Introduction

Several experimental studies have proven that inflammation occurs after intracranial hemorrhage (ICH), which is involved in brain injury [1]. Normally, microglia in brain tissue are activated when brain injury occurs. They become immune effector cells of the central nervous system. They are important sources and locations of cytokines in the brain and participate in pathological processes such as inflammation and injury [2]. Microglia mainly produce IL-1β, which counteracts the microglia to produce reactive oxygen species, resulting in oxidative damage [3]. In addition, Mayne et al. injected collagenase into the rat striatum to establish a model of cerebral hemorrhage. Four hours after collagenase injection, it was found that leukocyte-like cells around the hematoma contained immunoreactive anti-TNF-α antibodies, and microglia around the hematoma expressed TNF-α after being modeled for 8 h [4].

Eupatilin is an important flavonoid, and the active ingredient in the leaves of *Artemisia argyi*. The literature reports that Eupatilin treats diseases through various mechanisms, including anti-inflammatory [5,6], anti-cancer [7,8], and anti-apoptosis [9,10] effects. Song EH et al. found that Eupatilin suppresses the allergic inflammatory response in vitro and in vivo [11]. Yu K et al. found that Eupatilin protects against TNF-α-mediated inflammation in human umbilical vein endothelial cells [12]. Spakota A et al. reported that Eupatilin exerts neuroprotective effects in mice with transient focal cerebral ischemia by reducing microglial activation [13]. However, only one article has reported that

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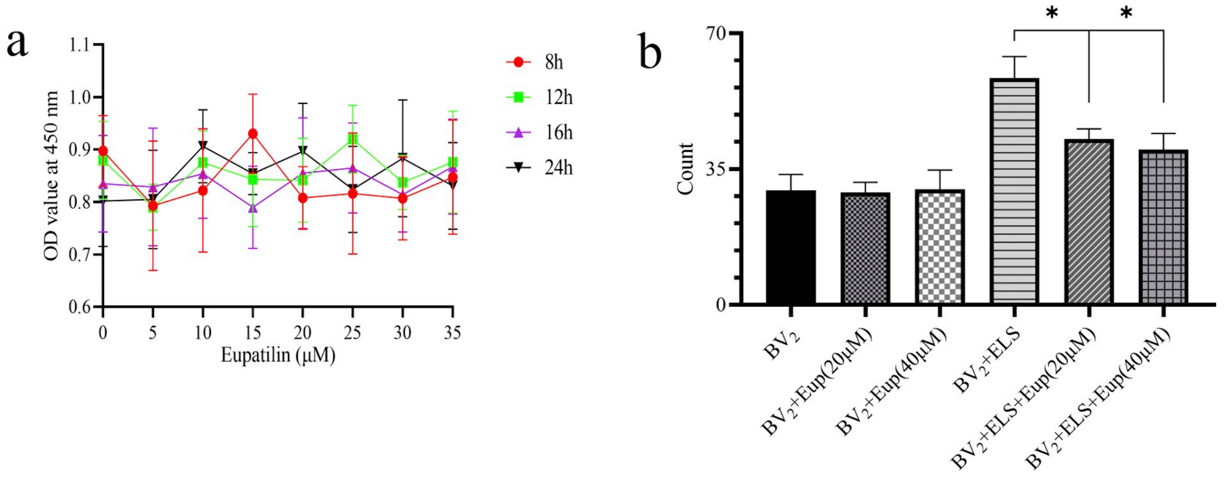

Fig. 1. Effect of Eupatilin on the viability and migration of microglia BV2.
a: BV2 cells were treated with different concentrations (0, 5, 10, 15, 20, 25, 30, 35 μM) of Eupatilin for different durations (8, 12, 16, 24 h), incubated with CCK-8, and the OD value at 450 nm was measured. b: After incubation of the BV2 cells for 24 h under different treatment conditions, the number of cells passing through the Transwell chamber was counted under a microscope. Data are expressed as mean ± standard deviation of three independent experiments. BV2 + ELS: 58.50 ± 4.77; BV2 + ELS + Eup (20 μM): 42.75 ± 2.78; BV2 + ELS + Eup (40 μM): 40.00 ± 3.61. *P < 0.05 vs. the control group.

Eupatilin inhibits microglia activation and attenuates brain injury in ICH [14].

Therefore, we used mouse BV2 cells for in vitro experiments to investigate whether Eupatilin inhibited ICH-induced inflammation and explored its possible anti-inflammatory mechanisms, which were validated in transgenic mice.

2. Materials and methods

2.1. Cell culture

The mouse BV2 cell line was purchased from the Chinese Academy of Medical Sciences (Beijing, People's Republic of China). The use of the cell lines was approved by the General Army Hospital institutional review board and ethics committee. BV2 cells were cultured in DMEM medium containing 10% fetal bovine serum. The incubator temperature was set to 37 °C and 5% carbon dioxide. The cells were subcultured 3 times a week. We treated the cells with erythrocyte lysate (1 μL of red blood cell lysate per mL of medium) to create an in vitro cerebral hemorrhage inflammation model. Cells were incubated for 24 h under different treatment conditions and used in different experiments.

2.2. Reagents

A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Chemical Technology Co., Ltd., Shanghai, China (Cat. No. CK04). Eupatilin was purchased from Sigma (Cat. No. SML1689). Red blood cell lysate was purchased from Solarbio Technology Co. Ltd., Beijing, China (Cat. No. R1010). Anti-TLR4 (Cat. No. ab13556), anti-MyD88 (Cat. No. ab2064), anti-IKB alpha (phospho S36) (Cat. No. ab133462), anti-IKK alpha (Cat. No. ab32041), anti-NF-κB p65 antibody (Cat. No. ab16502), anti-TNF-α (Cat. No. ab 183218), anti-IL-1β (Cat. No. ab9722), anti-IL-6 (Cat. No. ab229381), anti-COX2/cyclooxygenase (Cat. No. ab179800), anti-C3 (Cat. No. ab100999), anti-C5/C5a (Cat. No. ab194637), anti-iNOS (Cat. No. ab178945), anti-NeuN (Cat. No. ab177487), anti-GFAP (Cat. No. ab53554), anti-Iba1 (Cat. No. ab178847), mouse IL-1 beta ELISA kit (Cat. No. ab100704), and mouse TNF alpha ELISA kit (Cat. No. 100747) were purchased from Abcam. One Step Mouse Genotyping Kit (Cat. No. 101-01), ChamQ SYBR Color qPCR Master Mix (Low ROX Premixed) (Cat. No. Q431-02), and HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) (Cat. No. R233-01) were purchased from Vazyme Biotech Co., Ltd., Nanjing, China. Lipofectamine™ 3000 Transfection Reagent was purchased from ThermoFisher (Cat. No. L3000-015). The siRNA-TLR4-knock out was designed and purchased from GenePharma Co., Ltd., Shanghai, China. TLR4 primers, MyD88 primers, TNF-α primers, and IL-1β primers were designed by Wcgene Biotech Co., Ltd., Shanghai, China, and purchased from Sangon Biotech Co., Ltd., Shanghai, China.

2.3. Cell viability assay

BV2 cells were cultured in 96-well plates, containing 2000 cells per well. Different concentrations of Eupatilin (5, 10, 15, 20, 25, 30, 35 μM) were added to the cells, which were then cultured for 8 h, 12 h, 16 h, and 24 h. CCK-8 stock solution (10 μL) was added to each well and the plates were incubated in an incubator for 1–2 h; the optical density (OD) value at 450 nm was measured. The experiment was repeated three times.

2.4. Transwell assay

BV2 cells were cultured in a Transwell chamber and diluted to 5000/200 μL in medium with 10% FBS. FBS medium (600 μL, 10%) was added under the chamber and treated with red blood cell lysate and different concentrations of Eupatilin. The cells were incubated in the incubator for 24 h, fixed with 4% paraformaldehyde for 30 min, and stained with 0.5% crystal violet for 5 min, and the numbers of cells in four different fields of view were counted under a microscope. The experiment was repeated three times.

2.5. RT-PCR

BV2 cells were incubated with red blood cell lysate and Eupatilin for 48 h. We harvested cells and extracted RNA from the cells using the TRIzol method. Reverse transcription was performed according to the protocol in the HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) kit. qPCR was performed according to the protocol in the ChamQ SYBR Color qPCR Master Mix (Low ROX Premixed) kit. The primers for TLR4 were (forward) 5′-TGT TCC TTT CCT GCC TGA GAC-3′ and (reverse) 5′-GGT TCT TGG TTG AAT AAG GGA TGT C-3′. The primers for MyD88 were (forward) 5′-GGT TCT GGA CCC GTC TTG C-3′ and (reverse) 5′-AGA ATC AGG CTC CAA GTC AGC-3′. The primers for IL-1β were (forward) 5′-GCA ACT GTT CCT GAA CTC AAC T-3′ and (reverse) 5′-ATC TTT TGG GGT CCG TCA ACT-3′. The primers for TNF-

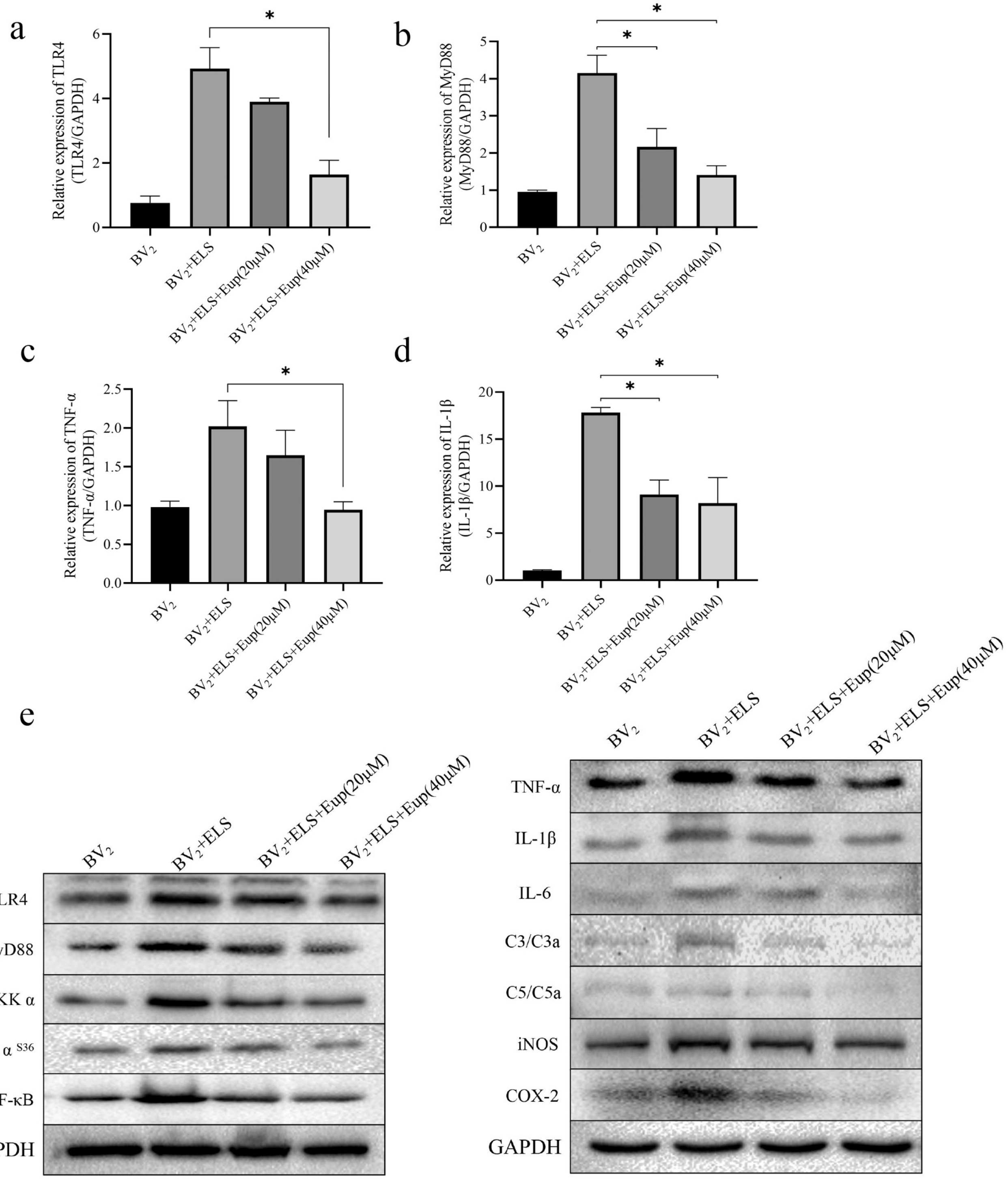

Fig. 2. Eupatilin inhibits the ELS-induced inflammatory response via the TLR4/MyD88 pathway.

Cells were incubated for 24 h under different treatment conditions and used for RT-PCR and western blotting. a: mRNA level of TLR4. BV2 + ELS: 4.93 ± 0.53; BV2 + ELS + Eup (40 μM): 1.64 ± 0.36. b: mRNA level of MyD88. BV2 + ELS: 4.15 ± 0.39; BV2 + ELS + Eup (20 μM): 2.16 ± 0.40; BV2 + ELS + Eup (40 μM): 1.41 ± 0.20. c: mRNA level of TNF-α. BV2 + ELS: 2.02 ± 0.27; BV2 + ELS + Eup (40 μM): 0.94 ± 0.08. d: mRNA level of IL-1β. BV2 + ELS: 17.82 ± 0.46; BV2 + ELS + Eup (20 μM): 9.12 ± 1.24; BV2 + ELS + Eup (40 μM): 8.19 ± 2.23. e: expression of TLR4, MyD88, NF-κB, IKK-α, IBKα^{S36}, TNF-α, Il-1β, IL-6, C3/C3a, C5/C5a, iNOS, COX-2. Data are expressed as the mean ± standard deviation of three independent experiments. *$P < 0.05$ vs. the control group.

α were (forward) 5′-GAT CGG TCC CCA AAG GGA TG-3′ and (reverse) 5′-CCA CTT GGT GGT TTG TGA GTG-3′. The primers for GAPDH were (forward) 5′-AGA GTG TTT CCT CGT CCC G-3′ and (reverse) 5′-CCG TTG AAT TTG CCG TGA-3′. The expression of related RNA was calculated by the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as a control. The experiment was repeated three times.

2.6. Western blotting

BV2 cell treated with erythrocyte lysate and Eupatilin were harvested in 1.5 mL EP tubes, 200 μL of protein extraction solution was added, and they were lysed on ice for 30 min; 50 μL of 5× SDS-PAGE loading buffer was then added for 10 min. The samples were

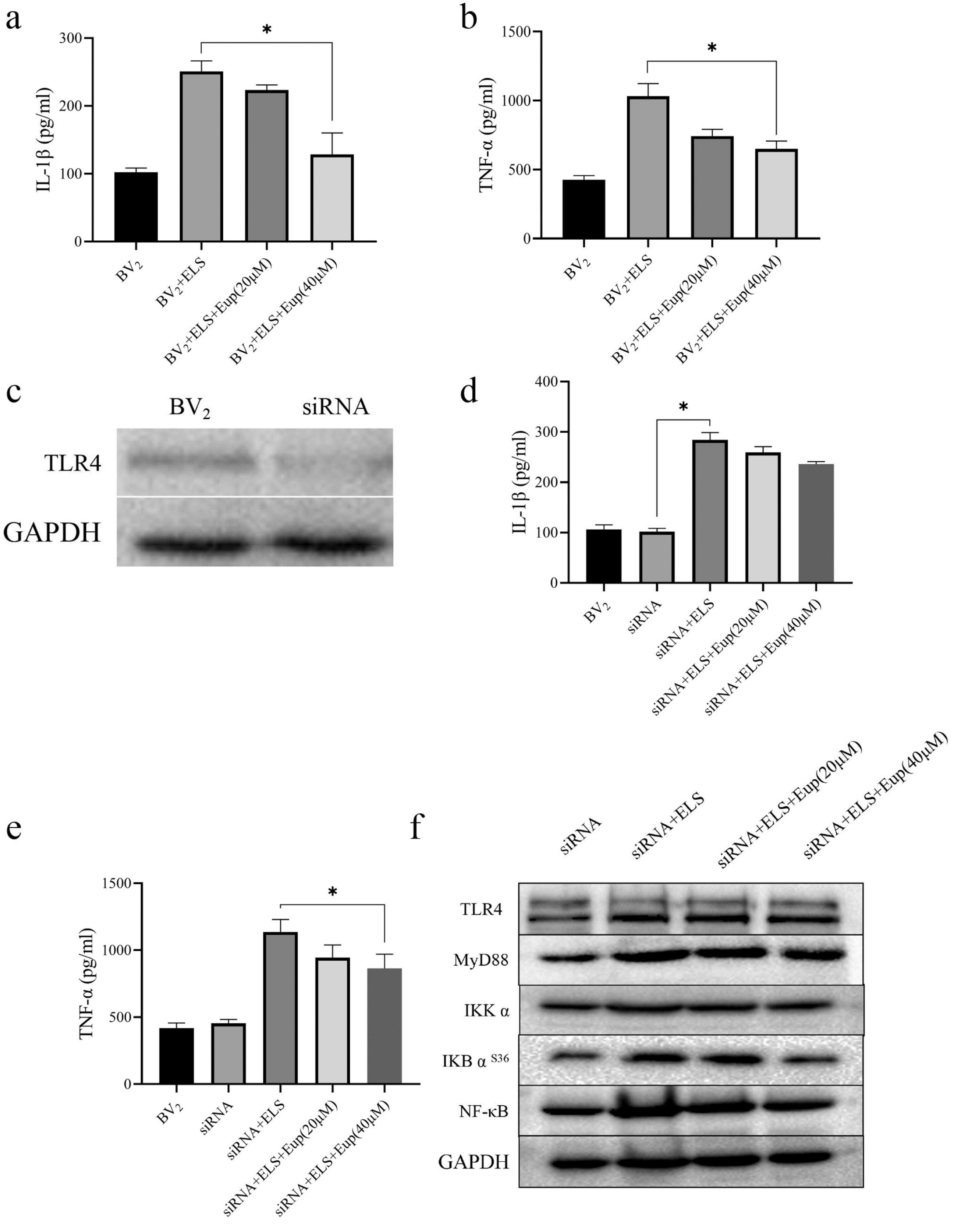

Fig. 3. Eupatilin inhibits the release of inflammatory cytokines by BV2, and the inhibition effect is attenuated by siRNA.

Cells were incubated for 24 h under different treatment conditions and their supernatants were used for the ELISA. a: IL-1β in cell supernatant. BV2 + ELS: 250.80 ± 12.80; BV2 + ELS + Eup (40 μM): 128.59 ± 25.71. b: TNF-α in cell supernatant. BV2 + ELS: 1031.43 ± 79.54; BV2 + ELS + Eup (40 μM): 651.19 ± 48.30. c: transfection efficiency was detected by western blotting after transfection with siRNA. After Eupatilin treatment of BV2 cells, which reduced TLR4 expression, the levels of the inflammatory factors IL-1β (d) (siRNA: 101.73 ± 5.52; siRNA + ELS: 284.12 ± 12.10) and TNF-α (e) (siRNA + ELS: 1137.37 ± 75.19; siRNA + ELS + Eup40 (μM): 864.70 ± 86.28) in the cell supernatant were examined. f: expression of TLR4, MyD88, NF-κB, IKK-α, and IBKα^{S36}. Data are expressed as the mean ± standard deviation of three independent experiments. *$P < 0.05$ vs. the control group.

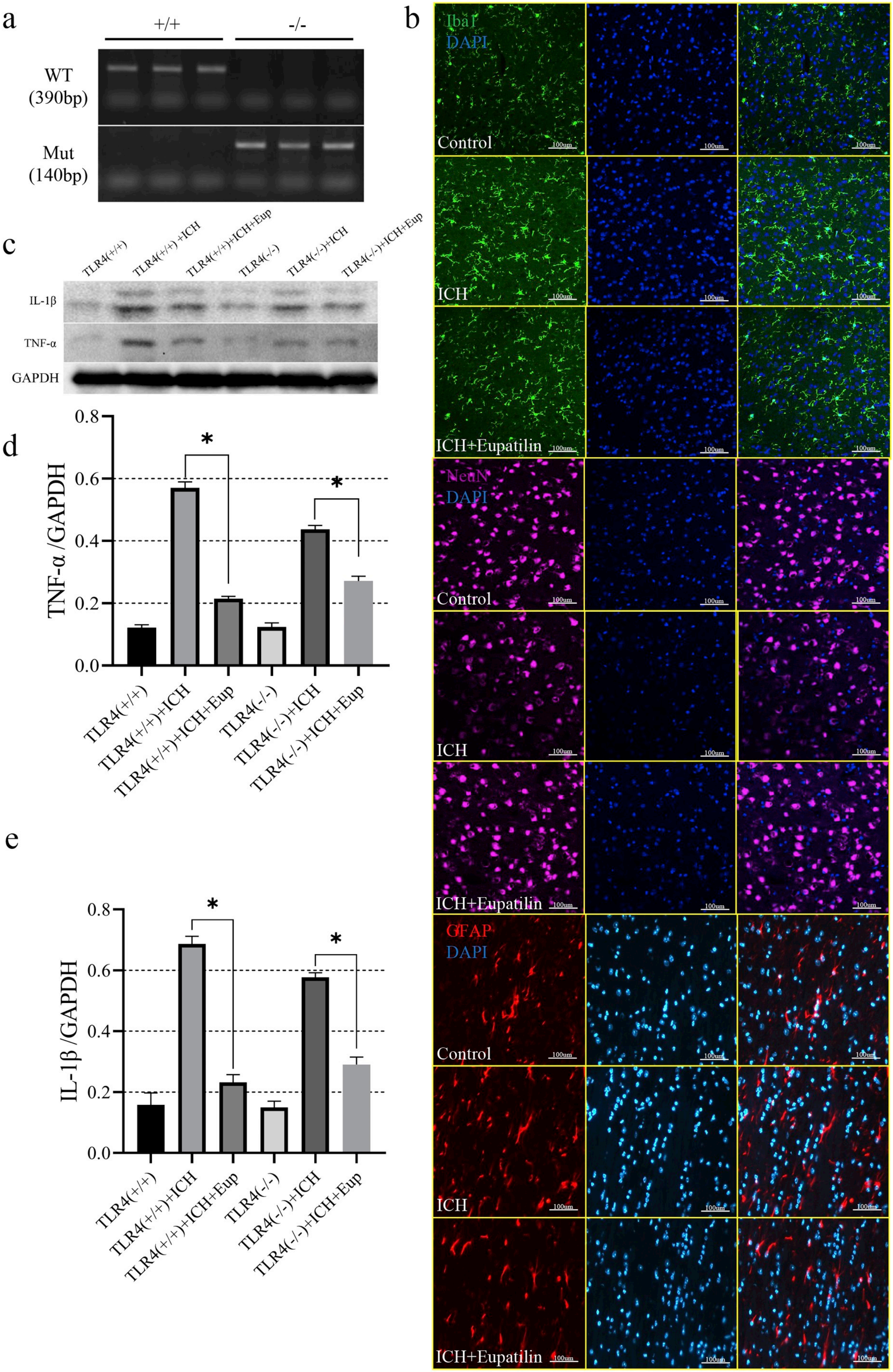

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Fig. 4. Eupatilin inhibits ICH-induced inflammation in vivo.
a: Genetic identification of transgenic mice by PCR. b: Immunofluorescence staining of the site with the largest cross-sectional area of bleeding in brain tissue from different groups. Wavelength: Iba-1: 488 nm. NeuN: 647 nm. GFAP: 555 nm. DAPI: 405 nm. c: TLR4 knockout mice and wild type mice were used to create an ICH model and treated with Eupatilin. Western blot analysis of IL-1β and TNF-α expression in the brain tissue around the hemorrhage site. d: Statistical analysis of IL-1β in panel c. TLR4 (+/+) + ICH: 0.570 ± 0.017; TLR4 (+/+) + ICH + Eup: 0.215 ± 0.006; TLR4 (−/−) + ICH: 0.437 ± 0.011; TLR4 (−/−) + ICH + Eup: 0.271 ± 0.013. e: Statistical analysis of TNF-α in panel c. TLR4 (+/+) + ICH: 0.686 ± 0.022; TLR4 (+/+) + ICH + Eup: 0.232 ± 0.021; TLR4 (−/−) + ICH: 0.578 ± 0.012; TLR4 (−/−) + ICH + Eup: 0.290 ± 0.021. Data are expressed as the mean ± standard deviation of three independent experiments. *$P < 0.05$ vs. the control group.

electrophoretically separated on a 12% SDS-PAGE gel (30 mg total protein/lane). After transfer to a PVDF membrane, the proteins were blocked with 5% BSA for 1 h and incubated overnight at 4 °C with the primary antibody (the dilution factor can be found on the official website of the antibody). The membranes were washed three times with TBST and then incubated with goat anti-mouse/ rabbit secondary antibody for 2 h at room temperature. After washing with TBST, the membranes were scanned by ECL and analyzed with the Bio-Rad gel imaging system.

2.7. ELISA

ELISA was performed after harvesting cell supernatants from different conditions for 24 h according to the protocol in the Mouse IL-1 beta ELISA Kit and the Mouse TNF alpha ELISA Kit. The OD value was read at 450 nm. The the standard curve equation and IL-1β and TNF-α content were calculated based on the OD value.

2.8. ICH model

All animal experiments were conducted according to protocols approved by the Institutional Ethics Committee of the Seventh Medical Center of the Chinese People's Liberation Army General Hospital. A total of 50 C57 mice were purchased from Shenzhen Huafukang Bioscience Co., Inc., (Shenzhen, China). Fifty TLR4-knock out C57 mice were purchased from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. Mouse genotypes were identified by PCR. PCR was performed according to the protocol in the One Step Mouse Genotyping Kit. The primers for TLR4-Mut were (forward) 5′-GCA AGT TTC TAT ATG CAT TCT C-3′ and (reverse) 5′-CCT CCA TTT CCA ATA GGT AG-3′. The primers for TLR4-Wt were (forward) 5′-ATA TGC ATG ATC AAC ACC ACA G-3′ and (reverse) 5′-TTT CCA TTG CTG CCC TAT AG-3′. Mice were anesthetized with 4% chloral hydrate (400 mg/kg) (intraperitoneal injection). Rectal temperature was maintained at 37.5 °C. The stereotactic technique was used to make a scalp incision along the midline and drill a burr hole on the left side of the skull (2 mm posterior and 1 mm lateral of the bregma). Collagenase (0.5 U/μL, 1 μL, Cat. No. C0773) was transferred into a 1 μL Hamilton syringe. The syringe was connected to a microinjection pump, and the needle was inserted into the brain through the burr hole (Depth: 2.8 mm from the bone surface). A total of 0.2 μL collagenase was injected over 10 min. After surgery, the skull hole was sealed with bone wax, the incision was closed with sutures, and the mice were allowed to recover. To avoid postsurgical dehydration, 0.5 mL of normal saline was administered to each mouse by subcutaneous injection immediately after surgery. Animals were sacrificed at different time points after ICH surgery for the indicated experiments.

2.9. HE staining

The brain tissue was frozen, sectioned, and placed in an oven at 37 °C for 10 min to dissolve the tissue-embedding agent and wash it. The staining step was as follows: Hematoxylin for 3 min, wash with water, hydrochloric ethanol for 10 s, wash with water, 1% ammonia for 10 s, wash with water, eosin for 1 min and 30 s, wash with water, 75% alcohol for 10 s, anhydrous alcohol for 10 s, xylene for 2 s, neutral resins mount, microscopic observation.

2.10. Immunofluorescence

Brain tissue was fixed with 4% paraformaldehyde for 48 h, dehydrated with 15% and 30% sucrose, and then frozen and sectioned. Sections were blocked with blocking buffer (1× PBS/5% FNS/0.3% TritonTM X-100) for 1 h at room temperature. The primary antibody was diluted with a dilution buffer (1× PBS/1% BSA/0.3% Triton X-100). Primary antibody was incubated overnight at 4 °C and then washed three times with PBS and incubated with fluorescent secondary antibody for 1 h at room temperature in the dark. They were then washed three times with PBS, mounted with DAPI, and observed under a confocal microscope.

2.11. Statistical analysis

All experiments were repeated three times and the data are expressed as mean ± standard deviation. The Shapiro-Wilk test was used to detect whether the data had a normal distribution. Two samples that conformed to the normal distribution were compared using the *t*-test. Multiple sample comparisons were analyzed by one-way ANOVA. Statistical analysis was performed using SPSS 17.0. *$P < 0.05$ was considered statistically significant.

3. Results

3.1. Eupatilin has no effect on BV2 cell activity and inhibits BV2 migration induced by ELS

The CCK-8 assay showed no significant change in the OD value at a wavelength of 450 nm of BV2 cells treated with Eupatilin (Fig. 1a). In addition, the Transwell experiments showed a significant increase in BV2 cells passing through the chamber in the presence of ELS. Eupatilin significantly attenuated the migration of BV2 cells induced by ELS, and this inhibitory effect is concentration-dependent (Fig. 1b).

3.2. Eupatilin inhibits the expression of inflammatory factors, which may be related to the TLR4/MyD88 pathway

Mouse microglia BV2 are the main source of inflammatory factors that must be studied to explore whether Eupatilin inhibits the inflammatory response caused by ELS. We examined the protein and mRNA levels of the intracellular inflammatory factors IL-1β and TNF-α after Eupatilin treatment and found that they were significantly increased by ELS. However, Eupatilin treatment significantly inhibited this increase in a concentration-dependent manner (Fig. 2c, d, e). The literature reports that triggering the Toll-like receptor 4 (TLR4) pathway often leads to the activation of NF-κB and the subsequent regulation of immune and inflammatory genes [15]. TLR4 interacts with a number of adaptor proteins in the cytoplasm (including the Toll/interleukin-1 receptor (TIR) domain), especially MyD88 [16]. NF-kB is a downstream effector of the TLR4/Myd88 signaling pathway regulated by IKK and IKB. Upon activation, it enters the nucleus to induce the synthesis of inflammatory factors. Therefore, we examined the transcription and translation levels of TLR4 and MyD88 and found a

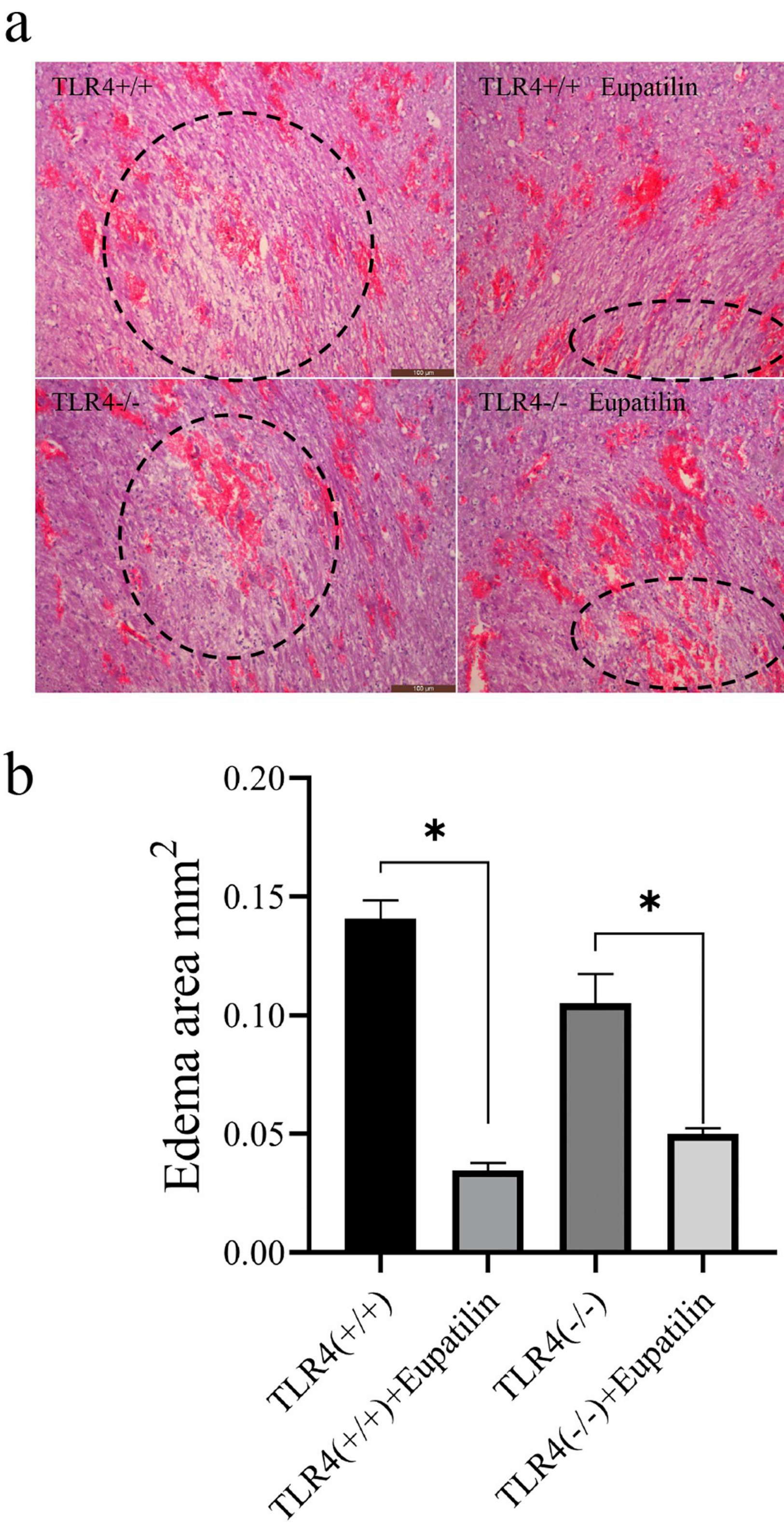

Fig. 5. Brain tissues from differently treated cerebral hemorrhage model mice were frozen, sectioned and HE stained. a: HE staining, the circled part represents edema. b: Quantification of the edema area (mm^2). Data are expressed as the mean ± standard deviation of three independent experiments. *$P < 0.05$ vs. the control group. TLR4 (+/+): 0.14051 ± 0.00682; TLR4 (+/+) + Eupatilin: 0.03482 ± 0.00247; TLR4 (−/−): 0.10506 ± 0.01076; TLR4 (−/−) + Eupatilin: 0.05008 ± 0.00207.

trending change consistent with induction of inflammatory factors (Fig. 2a, b, e). In addition, we examined the protein levels of NF-kB, IKK and phosphorylated IBK, which are downstream of TLR4 and other inflammation-related factors IL-6, C3/C3a, C5/C5a, iNOS, and COX-2. We found that ELS significantly increased the expression of these proteins, and Eupatilin treatment inhibited this increase (Fig. 2e). The levels of transcription and translation of intracellular IL-1β and TNF-α are regulated by multiple processes. Changes in inflammatory factors secreted by cells are more reflective of the anti-inflammatory effects of Eupatilin. We harvested the culture supernatant and performed an ELISA to detect the level of inflammatory factors in the supernatant. ELS was found to promote the release of inflammatory factors by BV2 cells, and Eupatilin attenuated their release (Fig. 3a, b).

3.3. siRNA attenuates TLR4 expression and inhibits the anti-inflammatory effect of Eupatilin

To further verify whether Eupatilin inhibits inflammation caused by ELS through the TLR4 pathway, we transfected siRNAs into the cells using Lipofectamine™ 3000 Transfection Reagent and verified transfection efficiency by western blotting. TLR4 in cells transfected with siRNA was significantly decreased (Fig. 3c). We found that cells transfected with siRNA showed a down-regulation of TLR4 expression, but the release of inflammatory factors by ELS was not significantly different from that of cells not transfected with siRNA. After treatment with 20 μM and 40 μM Eupatilin, respectively, it was found that both concentrations of Eupatilin slightly reduced the release of IL-1β after down-regulation of TLR4 expression (Fig. 3d). The difference was not statistically significant. In addition, we observed that 40 μM Eupatilin reduced TNF-α release (Fig. 3e) and the difference is statistically significant. However, compared with BV2 cells that were not transfected with siRNA (Fig. 3b), the effect of inhibiting the release of TNF-α was not significant. In addition, ELS still increased the expression of related proteins in the TLR4/MyD88/NF-kB signaling pathway after transfection of siRNA, and Eupatilin treatment failed to reduce the expression of these signaling proteins (Fig. 3f).

3.4. Eupatilin inhibits the inflammatory response caused by ICH in vivo

We use wild-type and TLR4-knockout mice to verify the results of in vitro experiments. We used PCR to identify genotypes in mice (Fig. 4a) and made ICH models. After the cerebral hemorrhage operation, mice with the same neurological score (Longa score) were grouped together (10 mice per group) and treated differently. Mice in the drug-treatment group were injected with Eupatilin (10 mg/kg, dissolved in physiological saline) immediately after injection of collagenase. On the third day post-operation, the mortality rates of each group of mice were: TLR4 (+/+): 0%; TLR4 (+/+) + ICH: 10%; TLR4 (+/+) + ICH + Eupatilin: 10%; TLR4 (−/−): 0%; TLR4 (−/−) + ICH: 0%; TLR4 (−/−) + ICH + Eupatilin: 0%. The mice were sacrificed to extract brain tissue around the bleeding site, which was frozen and sectioned after perfusion. To verify whether the target cells of Eupatilin in vivo are consistent with those in in vitro experiments, we stained for specific neuron, microglia, and astrocyte markers. We sliced and stained the site with the brain tissue with the largest cross-sectional area of bleeding in the different groups. We found that ICH induced activation of microglia and apoptosis of neuron in peripheral bleeding brain tissue and that Eupatilin inhibited the activation and apoptosis, but we did not observe significant changes in astrocytes (Fig. 4b). In addition, we found that ICH significantly increased IL-1β and TNF-α in brain tissue in both wild-type and TLR4-knockout mice. But wild-type mice had higher levels of inflammatory factors than knockout mice. In addition, the mice in the Eupatilin-treated group had significantly reduced IL-1β and TNF-α compared with the ICH model group. We observed that Eupatilin was less effective in TLR4 knockout mice than in wild-type mice (Fig. 4c, d, e).

3.5. Eupatilin inhibits brain edema and cell death caused by ICH in vivo

We selected ICH model mice with consistent behavioral scores for HE staining. Frozen sections and HE staining of mouse brain tissue was performed three days after creating the ICH model. We selected the site with the largest cross-sectional area of bleeding in the brain tissue from the different groups. We found that TLR4 knockout mice had less edema and cell death in their brain tissues after cerebral hemorrhage than wild-type mice. After treatment with Eupatilin, both groups had significant reduction in brain edema and cell death, but Eupatilin is more effective in treating brain edema in wild-type mice than TLR4−/− mice (Fig. 5a, b).

4. Discussion

Inflammation is the most common complication of ICH, which can cause severe brain edema and cell death and can lead to poor prognosis [1,17]. Activated microglia are the main cells that release inflammatory factors in brain tissue [18,19]. ICH induces microglia activation and recruitment around the damaged tissue, causing edema and inflammation of the brain tissue surrounding the hemorrhage. We used ELS to simulate cerebral hemorrhage in vitro. In Transwell experiments, we found that EIS significantly induced the migration of microglia BV2, which was significantly inhibited by Eupatilin treatment in a concentration-dependent manner. We also observed that microglia were activated after ICH, and that Eupatilin could effectively inhibit their activation, and verified that the target cells of Eupatilin were microglia in vitro.

Toll-like receptors are pattern recognition receptors in the body's natural immune system that recognize molecular patterns associated with different pathogens and initiate related downstream signaling pathways [20]. TLR4 contains a sequence-conserved domain TIR in the intracellular region, which recruits downstream adaptor proteins to activate downstream pathways, resulting in the massive release of inflammatory factors such as TNF-α and IL-1β [16,21]. The TLR4 pathway is closely associated with infectious diseases, tumors, and autoimmune diseases such as systemic lupus erythematosus [22], and is considered an important target. The intracellular signaling pathway of TLR4 includes the MyD88-dependent [23] and non-dependent pathways [24], both of which recruit downstream proteins and finally activate nuclear factor-κB to up-regulate the expression of inflammatory genes [25].

The NF-κB/Rel transcription factor complexes with the inhibitory IκB protein and is present in the cytoplasm in an inactive state [26]. The phosphorylation of IκBα, by the IκB kinase (IKK) complex at the Ser32 and Ser36 sites leads to its activation, followed by its proteasome-mediated degradation, which leads to the release of nuclear NF-κB and nuclear translocation [27,28]. Our results reveal that Eupatilin inhibits inflammation and also reduces the transcription and translation levels of TLR4 and MyD88, so we hypothesized that the anti-inflammatory mechanism of Eupatilin may be related to TLR4. We also examined the protein levels of NF-kB, IKK, and phosphorylated IBK and found that ELS significantly increased their expression, which was inhibited by Eupatilin treatment. To test our hypothesis, we down-regulated the expression of TLR4 using siRNA and found that the anti-inflammatory ability of Eupatilin was significantly reduced. Therefore, we believe that Eupatilin inhibits ELS-induced inflammation through the TLR4/MyD88 pathway.

We examined the expression levels of the classical inflammatory factors IL-1β and TNFα, and also measured the changes in IL-6, C3/C3a, C5/C5a, iNOS, and COX-2. IL-6 is a pleiotropic cytokine that regulates the acute phase response. IL-6 was originally designated as B cell differentiation factor, interferon beta 2, hepatocyte stimulating factor, cytotoxic T cell differentiation factor, and B cell stimulating factor-2; it promotes T cell population expansion and activation and B cell differentiation. In non-infectious inflammation such as that in burns [29] or traumatic injury [30], IL-6 can be produced by cells stimulated by Toll-like receptors. This acute IL-6 expression plays a central role in stimulating various cell populations in host defense. C3 and C5 play an important role in the activation of the complement system. This is a central response in the classical and alternative complement pathways, which is induced by C3/C5 convertase treatment. Degraded by the proteolytic degradation of complements C3 and C5, C3/C5 anaphylatoxin is the mediator of the local inflammatory process. It induces the contraction of smooth muscle, increases vascular permeability, and causes histamine to be released by mast cells and basophils. C5a also stimulates the movement of polymorphonuclear leukocytes and directs their migration to the site of inflammation. iNOS produces nitric oxide (NO). NO mediates bactericidal action in macrophages. INOS also has nitrosating enzyme activity and mediates cysteine S-nitrosylation of cytoplasmic target proteins such as COX2. Both iNOS and COX-2 play important roles in inflammation [31]. We found that Eupatilin can fully inhibit the expression of these inflammatory factor-related molecules and globally suppress microglia reactivity.

To further validate the results of our experiments in vitro, we performed experiments in vivo using TLR4 knockout mice. We used collagenase VII to make a mouse basal ganglia hemorrhage model, and in the treatment group, we injected Eupatilin in situ after injecting collagenase. HE staining showed that edema and hemorrhage in TLR4 knockout mice were significantly improved compared to that in wild type mice after injection with the same dose of collagenase. In addition, the levels of inflammatory factors in the brain tissue surrounding the hemorrhage were also significantly lower in TLR4 knockout mice. This suggests that TLR4 plays an important role in inflammation caused by ICH. In addition, Eupatilin treatment significantly improved the degree of inflammation in the brain tissue surrounding the bleeding. However, the therapeutic effect was not obvious in TLR4 knockout mice, indicating that part of the therapeutic effect of Eupatilin is exerted through the TLR4 pathway. Given the complexity of the in vivo environment, Eupatilin may also inhibit inflammation through the non-TLR4 pathway, and the underlying mechanisms remain to be further studied.

There are also some shortcomings in our experiments. The TLR4 knockout mice we used belong to deletion mutations in the type of gene mutation. Although the tail gene was identified by PCR, we were able to detect the TLR4 protein in western blot using the poly-clonal TLR4 antibody. This also indicates that the TLR4 knockout mouse used in our experiments is not a complete TLR4 deletion, and the mutant gene may also transcribe and translate proteins having certain structures similar to TLR4. Our experiments only illustrate the indirect association between Eupatilin and TLR4 and do not rule out the effects of TLR4-like proteins and Eupatilin. The direct interaction between Eupatilin and TLR4 remains to be further studied. In addition, we can clearly observe the boundary between the bleeding site and the brain tissue in the frozen section. Since the transmission electron microscopy requires pretreatment of the sample with osmium tetroxide, we cannot distinguish whether the cells observed under the microscope are derived from normal brain tissue or bleeding site. Therefore, we regret that we cannot verify the effect of Eupatilin on the ultrastructure of cells.

Overall, we found that injection with Eupatilin at the site of the bleeding significantly attenuated inflammation in the brain tissue surrounding the bleeding site. Clinically, the occurrence of cerebral hemorrhage is often unexpected. In many studies, treatment is administered intraperitoneally in advance of the cerebral hemorrhage. This mode of administration does not correspond to the actual situation of clinical patients. Our experiments were performed in situ with cerebral hemorrhage. On the one hand, this avoids the blocking of drugs by the blood-brain barrier. On the other hand, it more closely resembles the actual clinical situation. Patients with cerebral hemorrhage clinically consistent with surgical indications are prone to various complications caused by inflammation after surgery. In situ injection of therapeutic drugs better reflects its therapeutic effect and applicability. However, Eupatilin's toxicity and adverse effects on normal brain tissue remain to be further studied.

5. Conclusion

Our study found that Eupatilin inhibits ELS-induced inflammation in vitro and has a therapeutic effect on ICH-induced inflammation in vivo.

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