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Chapter

The Role of Microglia in Neuroinflammation

Shao-Wen Hung, Chia-Chi Chen, Hsiao-Yun Chen, Ying-Ching Hung, Ping-Min Huang and Chia-Yu Lin

Abstract

Microglia typically exist in a resting state of a mature brain and monitors the brain environment. In response to brain injuries or immunological stimuli, however, microglia are readily activated. In their activated state, they can serve diverse beneficial functions essential for enhancing neuron survival through the release of trophic and anti-inflammatory factors. Under certain circumstances, such as sustained epilepsy, however, microglia become overactivated and can induce significant and highly detrimental neurotoxic effects by the excessive production of a large array of cytotoxic factors, such as nitric oxide and proinflammatory cytokines. Neuroinflammation has been identified in epileptogenic tissue and is suspected of participating in epileptogenesis. Recent evidence has shown the effects of anti-inflammatory and protection against ischemic brain injury by inhibiting soluble epoxide hydrolase (sEH) pharmacologically and genetically. We assume that sEH inhibition might be also beneficial to prevent inflammatory processes caused by seizures and subsequent chronic epilepsy. In the present study, we investigated whether sEH is involved in overactivated microglia-induced neuroinflammation and subsequent epileptogenesis in a mouse model of temporal lobe epilepsy. Overactivated microglia will be detected by using imaging techniques. It is hoped that the results of the present study would provide a better understanding of the roles of sEH and microglia in epileptogenesis.

Keywords: epilepsy, epileptogenesis, microglia, neuroinflammation, soluble epoxide hydrolase

1. Introduction

Neuroinflammation has been identified in epilepsy-related tissue from both experimental and clinical evidence and is suspected to participate in the formation of neuronal cell death, reactive gliosis, and neuroplastic changes in the hippocampus, which may contribute to epileptogenesis [1–4]. The role of active microglia in neuroinflammation is tightly regulated under neurodegenerative processes. Therefore, the microglial regulation of neuroinflammation may provide a therapeutic target for the treatment of severe or chronic neuroinflammation (Figure 1).
During neuroinflammation, the pro-inflammatory-related cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), are produced by active microglia or astrocytes and next provoked pathological signaling cascades through phospholipase C and phospholipase A2 activations [5, 6]. Finally, the oxidized enzymes released the non-esterified arachidonic acid (AA) from cellular phospholipids and the formation of lysophospholipids and bioactive eicosanoids (Figure 2).

2. Epilepsy and treatments in people

In total, 1–3% of people in the world approximately suffer from epilepsy. Pharmacotherapy is the main treatment for most epileptic patients [7–10]. Moreover, the surgery is another option for epileptic patients according to the clinical doctors’ diagnosis by referring to brain imaging and seizure mapping techniques. When
epileptic patients cannot control seizures, by treatment with antiepileptic drugs (AEDs) or are not viable for surgery, vagal nerve stimulation will be a third possible option [11–16]. Unfortunately, a number of epileptic patients cannot control seizures. Herein, it is needed to research and develop more efficacious therapies for these epileptic patients with uncontrolled seizures [17–20].

3. The role of enzyme systems in the neuroinflammation

The cyclooxygenases, lipoxygenases, and cytochrome P450 (CYP) epoxygenases participated in metabolizing released AA to lipid metabolites as leukotrienes, epoxyeicosatrienoic acids (EETs), and prostaglandins (Figure 2). Brain parenchymal tissue metabolizes AA to EETs via the CYP epoxygenase, which regulates cerebral blood flow (CBF) and against neuroinflammation and apoptosis. Recently, hypoxia and ischemic preconditioning experiments have shown that the increased expression of CYP epoxygenase and EETs in the brain may confer protection from an ischemic stroke induced by middle cerebral artery occlusion (MCAO) in the animal model. It also suggests that EETs signaling may suppress the ischemia-evoked inflammatory cytokine response in the brain, supporting an anti-neuroinflammatory role for EETs in the brain circulation [21–28].

Iba1 is specifically expressed in microglia or macrophages and is up-regulated during the activation of these cells following nerve injury, central nervous system ischemia, and several other brain diseases. Additionally, whether soluble epoxide hydrolase (sEH) expression in the microglia should be verified? sEH can perform the metabolic conversion of EETs into their less active form as dihydroxyeicosatetraenoic acids. Currently, the inhibition of sEH has been used to increase systemic EETs level and bioactivity. Through applying the pharmacologic inhibitors or genetic deletion, the inhibition of sEH attenuated the cerebral ischemia-induced vascular and neural injury, suggesting sEH might be a novel target for stroke treatment [29–37].

4. Experimental design in vitro for evaluating the role of microglia in Neuroinflammation

The reagents were ordered and prepared to perform in vitro experiment. The 12-(3-Adamantan-1-yl-ureido)-dodecanoic acid (AUDA) was ordered from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO; Cat No. 472301; Sigma-Aldrich, MO, USA). Pilocarpine was ordered and dissolved in 0.9% saline. The 90% ethanol (Sigma-Aldrich), Liu’s stain (ASK, Taoyuan, and Taiwan), and Griess reagent system (Promega, Madison, and WI) were ordered. Cytofix/Cytoperm™ (BD Biosciences, CA, USA), Perm/Wash™ (BD Biosciences), mouse anti- Iba1 monoclonal antibody (sc-52,328; Santa Cruz Biotechnology), mouse anti-sEH monoclonal antibody (sc-6260; Santa Cruz Biotechnology), and FITC-labeled goat anti-mouse IgG antibody (1:1000) (sc-2010; Santa Cruz Biotechnology) were ordered for the determination of activated microglial marker and sEH expression by flow cytometric assay. The 3-(4,5-Dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, MERCK, Darmstadt, Germany) was ordered and dissolved in 1× phosphate-buffered saline (PBS; Sigma-Aldrich).

Mouse retroviral immortalized microglia BV-2 cells belonging to the C57BL/6 background were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented
with 10% fetal bovine serum (FBS; Gibco®), 2 mM L-glutamine, 100 U/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich) in 5% CO₂ atmosphere at 37°C. Cells were treated with 100 μM pilocarpine and/or 100 μM AUDA and cultured for 24 hrs in 10% FBS-DMEM on glass coverslips. Observation of cell morphology with/without treatment was done by light microscope (Olympus CKX41, Olympus Optical Co. Ltd.). Cells were grown to 90% confluency before the experiments.

Measurement of cell viability was measured by MTT assay according to the manufacturer's instructions. At the experimental points, cell viability was detected by MTT assay. The reduced purple dye intensity of color was estimated by reading at an optical density of 570 nm in a spectrophotometer. Moreover, In vitro migration assays (scratch assay and transwell migration assay) were performed. Scratch assays were performed following the previously described. Briefly, the BV-2 microglia in six well plates were performed with serum-free DMEM for three wash times. A line down the center of each well was scraped with a sterile p200 pipette tip and followed by a wash step to remove debris with serum-free DMEM. Images were taken at 10× magnification of the light microscopy (Olympus BX43, Olympus Optical Co. Ltd.). The scratch widths were measured and wound closure was calculated by dividing widths measured after 8 hours of incubation by the initial scraped width. Each experiment was carried out in triplicate and three fields were blindly counted per well by scorers. Transwell migration assays were performed by using Boyden chambers (BD Bioscience). The 4 × 10⁴ BV-2 microglia (200 μL serum-free DMEM) were added to the upper chamber of Boyden chambers and allowed to adhere to the polycarbonate filters (8 μm pore) for 30 mins at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Following this, BV-2 microglia were treated with 100 μM pilocarpine at 37°C for 30 mins prior to AUDA treatment. The 100 μM AUDA was then placed in the upper chamber and the lower chamber was added with 10% fetal bovine serum (FBS)-DMEM to attract BV-2 microglia migration. BV-2 cells did not migrate and remained on the upper surface of the Boyden chambers’ filter were removed. BV-2 cells that had migrated to the lower surface were fixed with 90% ethanol, stained with Liu’s stain (ASK, Taoyuan, Taiwan), and counted. In at least three independent experiments, three wells per treatment were blindly counted in nine random fields at 40× magnification per well by scorers.

A phagocytosis assay was performed in this experiment. BV-2 microglia seeded in six well plates were incubated with 100 μM pilocarpine at 37°C for 30 mins prior to 100 μM AUDA treatment for 24 hrs. After 24 hrs treatment, the phagocytic ability of the microglia was measured by using FITC-labeled dextran (MW 40,000) as a tracer. Briefly, microglia were exposed to 30 μg/mL FITC-labeled dextran for 30 mins. Later, three washing times with cold PBS (pH 7.4) were performed prior to measuring fluorescence at 480 nm excitation and 520 nm emission on a flow cytometer (FACS Calibular, BD Biosciences) or fluorescence microscopy (Olympus BX43, Olympus Optical). As a background, the cultures without FITC-dextran were used. Each culture condition was performed in quadruplicate, and three independent experiments were performed.

Measurement of extracellular nitric oxide production was performed. The nitrite, a stable breakdown product of nitric oxide, was measured with a Griess Reagent System (Promega, Madison, WI). Determination of activated microglial marker and sEH expression by flow cytometric assay was performed. First, BV-2 cells were pre-treated with 100 μM pilocarpine for 30 mins then were treated with 100 μM AUDA for 24 hrs in 10% FBS with DMEM. After pilocarpine-AUDA co-treatment, these cells were harvested and fixed in Cytofix/Cytoperm™ (BD Biosciences) at 4°C for 15 mins and washed twice with 1× Perm/Wash™ (BD Biosciences). Fixed cells were stained with various primary antibodies [mouse anti-Iba1 monoclonal antibody (1:100...
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dilution) (sc-52,328; Santa Cruz Biotechnology) and mouse anti-sEH monoclonal antibody (1:100 dilution) (sc-6260; Santa Cruz Biotechnology)) at 4°C for 30 mins and then washed twice with 1× Perm/Wash™ (BD Biosciences). Secondary antibodies [FITC-labeled goat anti-mouse IgG antibody (1:1000 dilution) (sc-2010; Santa Cruz Biotechnology)] were subsequently stained at 4°C for 30 mins. Finally, cells were stained with 5 μg/mL PI (propidium iodide; BD Biosciences) at room temperature for 5 mins. Cells were analyzed by a flow cytometer (FACSCalibur, BD Biosciences) and WinMDI software (version 2.9). Statistical analysis was performed in this study. The values are reported as mean ± SE. All statistical comparisons were made with two-tailed tests. Statistical evaluation was performed using Student’s t-test, one-way ANOVA, and/or Dunnett’s post hoc test. Differences between groups were considered statistically significant at **p < 0.01 and ***p < 0.001.

5. AUDA significantly inhibited pilocarpine-induce BV-2 microglial activation and cytokine expressions

The 100 μM pilocarpine did not affect cell viability and the half-maximal inhibitory concentration (IC₅₀) was 17.5 mM. The 100 μM AUDA did not affect cell viability and the

![Figure 3](image_url)

**Figure 3.** BV-2 microglial cell viability for the pilocarpine and/or AUDA treatment. (A) BV-2 cells treated with the serial two-fold diluted concentration of pilocarpine (0 to 100,000 μM) at 37°C for 24 hrs in 10% serum-DMEM. Cell viability was determined by using MTT assay. The half maximal inhibitory concentration (IC₅₀) of pilocarpine was 17.5 mM. (B) BV-2 cells treated with the serial two-fold diluted concentration of AUDA (0 to 16,000 μM) at 37°C for 24 hrs in 10% serum-DMEM. Cell viability was determined by using MTT assay. 100 μM pilocarpine did not affect cell viability and the half maximal inhibitory concentration (IC₅₀) of AUDA was 0.35 mM. (C) Non-cytotoxic concentration (100 μM) of pilocarpine and AUDA were used in this study. Non-cytotoxic effect was presented after 100 μM pilocarpine combined with 100 μM AUDA treatment at 37°C for 24 hrs in 10% serum-DMEM. Values are reported as mean ± SE.
half-maximal inhibitory concentration (IC₅₀) was 0.35 mM. Non-cytotoxic concentration (100 μM) of pilocarpine and AUDA were used in this study (Figure 3A and B). The non-cytotoxic effect was presented after 100 μM pilocarpine combined with 100 μM AUDA treatment (Figure 3C). The mean fluorescence intensity (MFI) of Iba1 expression was significantly increased in the BV-2 microglial cells under direct 100 μM pilocarpine stimulation. AUDA significantly decreased Iba1 expression. sEH expression was presented in the BV-2 microglia. 100 μM AUDA significantly decreased sEH expression in BV-2 microglia. AUDA significantly suppressed pilocarpine-active BV-2 cell migration by using wound-healing assay. AUDA significantly suppressed pilocarpine-active BV-2 cell migration by using Boyden chamber assay. Histogram showed 100% phagocytosis in all groups. AUDA significantly suppressed phagocytic abilities of pilocarpine-active BV-2 cells by using flow cytometry. No effects of nitric oxide production were presented in all groups. Values are reported as mean ± SE. All statistical comparisons were made with two-tailed tests. Statistical evaluation was performed using Student’s t-test. Differences between groups were considered statistically significant at *p < 0.05; **p < 0.01; ***p < 0.001.
stimulation (Figure 4A). The sEH expression was presented in the BV-2 microglia (Figure 4B). C-terminal inhibitor of she, AUDA (100 μM), significantly decreased Iba1 and sEH expressions in the active BV2 microglia (Figure 4A and B). After microglial activation, cell migration, phagocytosis, and cytotoxicity were enhanced. According to these results, AUDA significantly suppressed cell migration, and phagocytosis (Figure 4C–H). Additionally, alone or combined pilocarpine or AUDA treatment did not affect extracellular nitric oxide production in BV-2 microglia (Figure 4I).

6. Discussion

Epilepsy affects approximately 1–3% population of the world, and temporal lobe epilepsy (TLE) is the most common localized epilepsy disorder, accounting for approximately 40% of adults with epilepsy [38]. However, the exact mechanism for the formation of TLE remains unclear. According to the engulfment-promoted cell death theory, nerve cells have special receptors. When nerve cells are injured, activated microglia will recognize this receptor and contact nerve cells, indirectly causing nerve cell death [39]. In addition, some studies have confirmed that microglia can also be directly activated by some activating factors, thereby affecting the function and survival of nerve cells [39]. Previous studies have confirmed that the EETs-sEH pathway is associated with brain inflammation, but whether the EETs-sEH pathway is involved in the formation of TLE remains to be clarified. For these reasons, studying the molecular and cellular mechanisms of the brain’s transition from normal to epilepsy can be used to understand the neurobiological changes in epilepsy formation and provide a new therapeutic strategy. Therefore, this study hopes to find a new treatment by understanding the performance and function of sEH microglia in the resting state and the microglia in the activated state, and using the functional inhibitor of sEH to find out how to regulate the activation of microglia. The method of epilepsy is expected to provide clinicians with a reference for the treatment of epilepsy and the use of AEDs in the future. This study demonstrated that AUDA, an inhibitor of sEH, significantly inhibited sEH expression and pilocarpine-induced microglia activation, including phagocytosis and migration. From these results, pilocarpine can directly activate microglia, and inhibition of the EET-sEH pathway can inhibit activated microglia, including phagocytosis and migration. Based on these research results, it is hoped that in the future, it will be helpful to neuroscience researchers in molecular and cellular research on the pathogenic mechanism of TLE, and provide clinicians with a reference for treating epilepsy and the use of anti-epileptic drugs.

7. Conclusions

Neurological disorders are complicated in the brain and spinal cord and are caused by a loss of neurons and glial cells in these injured areas. Currently, neurological disorders can affect hundreds of millions of people worldwide. More than 50 million people have epilepsy worldwide. The microglia are a key causative factor in the process of neuroinflammation. Commonly, microglia are activated after the brain injury and the activated microglia can induce neurocytotoxic factors. At present, much evidence have demonstrated microglial activation following pilocarpine-induced seizures. Our results suggest a role for sEH in regulating epileptogenesis of
BV-2 microglia in vitro, whereas the effect of hydrolase inhibition on epileptogenesis may provide a novel therapeutic approach for approximately 20–40% of the clinically anti-epileptic drugs-uncontrolled epileptic patients.

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Conflict of interest

The authors declare no conflict of interest.

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