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Original Article

Neuroprotective effect of endogenous cannabinoids on ischemic brain injury induced by the excess microglia-mediated inflammation

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Abstract: Increasing evidence has demonstrated the role of endogenous cannabinoids system (ECS) on protecting brain injury caused by ischemia (IMI). Papers reported that microglia-mediated inflammation has become one of the most pivotal mechanisms for IMI. This study was aimed to investigate the potential roles of ECS on neuron protection under microglia-mediated inflammation. Inflammatory cytokines level both in vitro (BV-2 cells) and in vivo (brain tissue from constructed IMI model and brain-isolated microglia) was detected. ECS levels were detected, and its effects on inflammations was also analyzed. Influence of microglia-mediated inflammation on neuron injury was analyzed. Moreover, the effects of ECS on protecting neuron injury were also analyzed. Our results showed that the levels of inflammatory cytokines including TNFα and IL-1β were higher while IKBα was lower in IMI model brain tissue, brain-isolated microglia and BV-2 cells compared to the control. Inflammation was activated in microglia, as well as the activation of ECS characterized by the increasing level of AEA and 2-AG. Furthermore, the activated microglia-mediated self-inflammation performed harmful influence on neurons via suppressing cell viability and inducing apoptosis. Moreover, ECS functioned as a protector on neuron injury though promoting cell proliferation and suppressing cell apoptosis which were caused by the activated BV-2 cells (LPS induced for 3 h). Our data suggested that ECS may play certain neuroprotective effects on microglia-mediated inflammations-induced IMI through anti-inflammatory function.

Keywords: Endogenous cannabinoids, microglia, excess inflammation, ischemic brain injury

Introduction

Ischemic brain injury (IMI) also called as stroke, is a kind of cerebral blood circulation disorder characterized by clinical sudden faint flapping, unconscious, sudden strong mouth eye askew, hemiplegia or mental retardation [1]. A statistic report from the American Heart Association shows that the morbidity and mortality for IMI are high around the world [2]. Previous evidence shows that pathogenic factors for IMI are diversity, such as hypertension, diabetes, blood lipid metabolism or age and gender, leading to a high risk or huge economic burden for both patients and society [3, 4]. Therefore, to explore several new treatment drugs will be benefit for the clinical cure of IMI.

Cerebrum is a highly glucose- or oxygen-dependent neuron system, and hypoxia-caused ischemia brings huge damage to neuron system [5]. In recent years, the microglia-induced inflammations, which is a kind of macrophage, has become one of the most serious mechanism for IMI. The microglia secrete cytokines including NO, IL-1β, activated NF-κB and TNFα performed toxicity on neurons via different signal pathways, including directly combining with neurons or other inflammatory mediation toxicity [6, 7]. For example, the phosphatide serine residues on neuron membrane induced the phagocytosis of activated microglia in the early stage during neuron damage, leading to the neuron injury [8]. The microglia secreted IL-1β performed neuron injury through combining with N-methyl-

D-aspartate receptor, while TNFα was involved in the neuron damage via degrading myelin or enhancing intercellular adhesion molecules 1 expression in astrocytes [9, 10].

Endocannabinoid system (ECS) is involved in a wide range of physiological processes, including neurotransmission, mood, appetite, pain appreciation, addition behavior, and inflammation [11]. Anadamine (AEA) and 2-arachidonoyl glycerol (2-AG) are two proven ECs, which can act on the cannabinoid receptors, CB1 and CB2 [12]. Accumulating reports have showed the potential function or correlation between ECS and brain diseases, including cerebral palsy, stroke and other kinds of central nervous system and peripheral processes [13]. In spite of vast researches have explored the potential roles of ECS in brain injury. However, few studies have mentioned the correlation between ECS and IMI under the activated microglia induced-inflammation.

In this study, we analyzed the potential effects of ECS on IMI under microglia-mediated inflammation both in rabbit and in BV-2 cells. Inflammatory cytokine levels including IL-1β and TNFα were detected to verify whether IMI model was successfully constructed. Consequently, inflammations were analyzed both in rabbit brain, brain isolated microglia, as well as BV-2 cells. The protective effects of ECS on neuron cells were analyzed by detecting cell apoptosis and proliferation. This aim of study was to investigate the potential regulate mechanism of body on this inflammation under physiological condition, and to illustrate the potential effects of ECS on IMI under inflammation. Our study may provide theoretical basis for exporting potential treatment drugs for IMI in clinical.

Experimental procedures

Rabbit model

All the experimental procedures were approved by the relevant local research animal ethics committee. A total of 16 Japanese male white rabbits (weighting at 2.0 kg-2.5 kg) were randomly separated into two groups (8 rabbits in each group), which was defined as group A (sham) and group B (IMI). Treatment for rabbits in group B was as follows: rabbits were intraperitoneal injected with 10% chloral hydrate (0.0035 mL/g) for anesthesia. The left external carotid artery was exposed under surgery microscope for the ligation of telecentric position of external carotid artery, and the wedge incision was made on the starting position on stump site of the free external carotid artery. Setline (heating the head of setline to make the head to be a round ball, whose diameter is about 0.3 mm) was inserted into internal carotid artery from the bifurcate position of external carotid artery, for the block of blood supply in brain meddle artery. Rabbits in group A (sham) were treated similarly to that in group B except for the setline insertion. After wake up, rabbits characterized with the same side Horner characteristics and mainly forelimbs-oriented hemiplegia were considered as the symbol for the successful IMI model construction. Blood sample was isolated from the constructed IMI rabbit model at different time point for further research.

Cell culture

Murine microglia BV-2 cells were cultured in DMEM medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

Enzyme-linked immune sorbent assay (ELISA)

For inflammatory cytokines detection in IMI rabbit blood, ELISA was performed as previously described [14]. Briefly, the diluted antibody solution was incubated with blood samples for 1 h. After samples were washed with PBS buffer for 4 times, horseradish-peroxidase (HRP) solution was added into the samples and incubated for 1 h at 37°C. Consequently, tetramethylbenzidine (TMB) was added into samples for another incubation of 15 min in dark place at room temperature for the reaction block. Absorbance at 450 nm was detected using microplate reader (BioRad).

Western blot

Samples were lysised in RIPA assay (radioimmunoprecipitation, Sangon Biotech, China) lysate containing phenylmethanesufonyl fluoride (PMSF, Sigma, USA), cell lysates were collected and centrifuged for proteins collection. For western blot, 30 μg protein per lane was subjected onto a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-

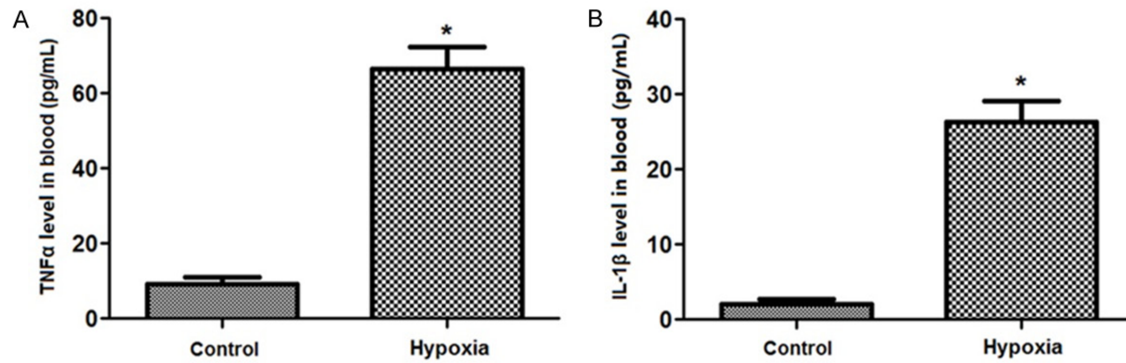

Figure 1. Inflammatory cytokines secretion in rabbit blood. A. TNFα level was significantly increased compared to the control group; B. When rabbit was exposed to hypoxia, IL-1β level in blood was significantly increased compared with that in control. *: P<0.05 compared to the control.

PAGE), followed by transferred onto a Polyvinylidencefluoride (PVDF) membrane (Mippore). The PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Consequently, the membranes were incubated with rabbit anti-human antibodies (IKBα, 1:100 dilution, Invitrogen, USA) and overnight at 4°C. Then the membranes were incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, PVDF membrane was washed with 1×TBST buffer 3 times for 10 min. Detection was conducted using the development of X-ray after chromogenic substrate with an enhanced chemiluminescence (ECL) method. Additionally, β-actin (Sigma) served as the internal control.

LC-MS-MS

Cells at density of 8×10^6/mL were injected into 75 cm^2 culture flask and overnight at 37°C. Then cells were treated with 1% O_2 for 6 h and 12 h respectively. Collected cells were washed with PBS buffer for 3 times, and then resuspended in 10 mL PBS buffer for ultrasonic disruption. Consequently, disrupted cells were mixed with 200 μL methanol/H_2O (v:v, 2:1) and 3 mL acetidin-N-hexane (v:v, 9:1), mixtures was then vibrated at 240 times/min for 15 min. After that, mixtures were centrifuged at 3500 r/min for 5 min to obtain the lower organic phase. Residue was resuspended with 100 μL methanol. Finally, 20 μL sample was detected using Agilent 1100 HPLC system and API4000 Triple tandem quadrupole mass spectrometer (Applied Biosysterm, USA). Internal controls for AEA and 2-AG were purchased from Sigma. Detection for 2-AG was m/z 397.2 and for AEA was m/z 348.2.

MTT assay

Cell viability was performed using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay as previous described [15]. Briefly, after 96 h of incubation, 20 μL MTT was added into cells for another 4 h incubation. Then 150 μL dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min to stop the reaction. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

Cell apoptosis assay

Terminal deoxynucl neotidyl transferase mediated dUTP nick (TUNEL) assay was used to assess the apoptotic cells in each group according to the manufacturer's instructions [16]. Cells were plated on 40 mm round glass cover slips in 60 mm culture dishes. Then cells were fixed with 4% paraformaldehyde in PBS buffer. Nuclear staining was determined via green unclear fluorescence and was observed through laser scanning confolcal microscopy in more than 300 cells. Cells were observed in five fields, and cells with brown color were considered as positive cells while others were negative cells. Percentage of myocardial cell apoptosis was calculated as the following formula: 100%×total positive cells in 5 fields/total cells in 5 fields.

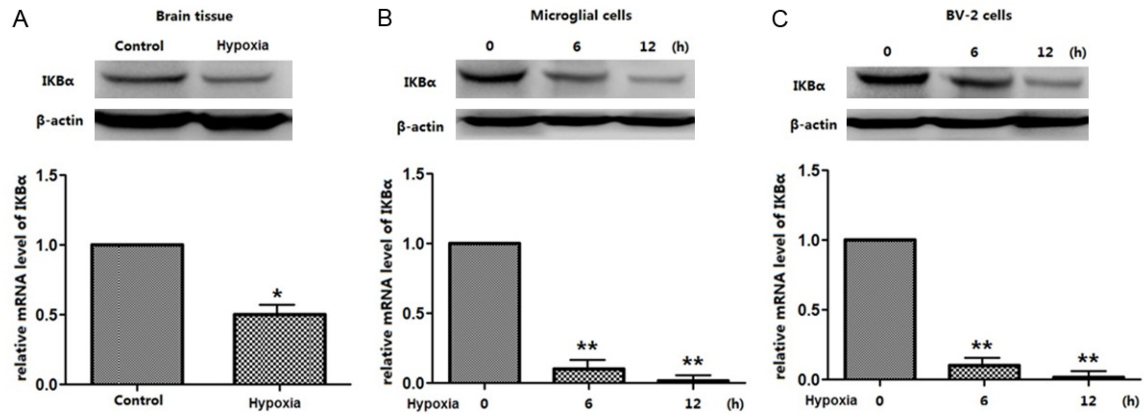

Figure 2. Secretion detection of IKBα in rabbit brain tissue, microglia and BV-2 cells. A. When rabbit was exposed to hypoxia, IKBα relative mRNA and protein level were significantly decreased compared to the control; B. When microglial cells were treated with hypoxia, IKBα relative mRNA and protein level were both significantly decreased with time increasing compared with that in cell at 0 h; C. Similar tendency of IKBα level change in BV-2 cells was observed. *: P<0.05, **: P<0.01 compared to the control.

Statistically analysis

All experiments were independently conducted for 3 times. Data are expressed as mean ± standard error of mean (SEM). Independent t-test or analysis of variance (ANOVA) was used to calculate the difference between two groups using SPSS 16.0. The P<0.05 was defined as statistically significant.

Results

Inflammatory factor levels in IMI rabbit blood

To verify whether the rabbit IMI model was successfully constructed, the levels of inflammatory factors including TNFα and IL-1β were detected (**Figure 1**). Compared to the controls, both TNFα and IL-1β levels were significantly increased (P<0.05), indicating the activation of inflammatory signals. Namely, the IMI rabbit model was successfully constructed.

IKBα secretion in rabbit brain tissue, microglia and BV-2 cells

NF-κB activation is positively correlated with inflammatory cytokine secretion including TNFα and IL-1β, namely, the highly secreted TNFα and IL-1β will promote NF-κB activation [17]. The major function of IKBα is interfering the intracellular combination between NF-κB and DNA [18]. Therefore, we further measured the secretion levels of IKBα in rabbit brain tissue, microglia and BV-2 cells to verify the inflammatory reaction (**Figure 2**). When brain tissue or cells was exposed to hypoxia, IKBα was significantly decreased compared to the control group (P<0.05). Also, its level in microglia or BV-2 cells was drastically decreased with time increasing than that in control (P<0.01), suggesting that the activation of inflammatory reaction in different tissue or cells.

AEA and 2-AG levels in BV-2 cells under the condition of hypoxia and LPS

To investigate whether the endogenous cannabinoids was correlated with hypoxia- or LPS-induced IMI, the secretion levels of AEA and 2-AG were detected using HPLC analysis (**Figure 3**). When BV-2 cells were exposed with hypoxia, both 2-AG and AEA levels were higher with time increasing than that in cells at 0 h (**Figure 3A** and **3B**). Besides, when cells were induced with LPS, both the 2-AG and AEA levels were all increased with time increasing (**Figure 3C** and **3D**). These results indicated that the endogenous cannabinoids system was activated in BV-2 cells.

The activated endogenous cannabinoids suppressed inflammations in BV-2 cells

We further investigated whether endogenous cannabinoids was correlated with inflammations in BV-2 cells, IKBα level in BV-2 cells which were treated with 2-AG and LPS or hypoxia was detected (**Figure 4**). The results showed that when BV-2 cells were treated with either hypoxia or LPS, the mRNA and protein level of IKBα was significantly inhibited by 2-AG application

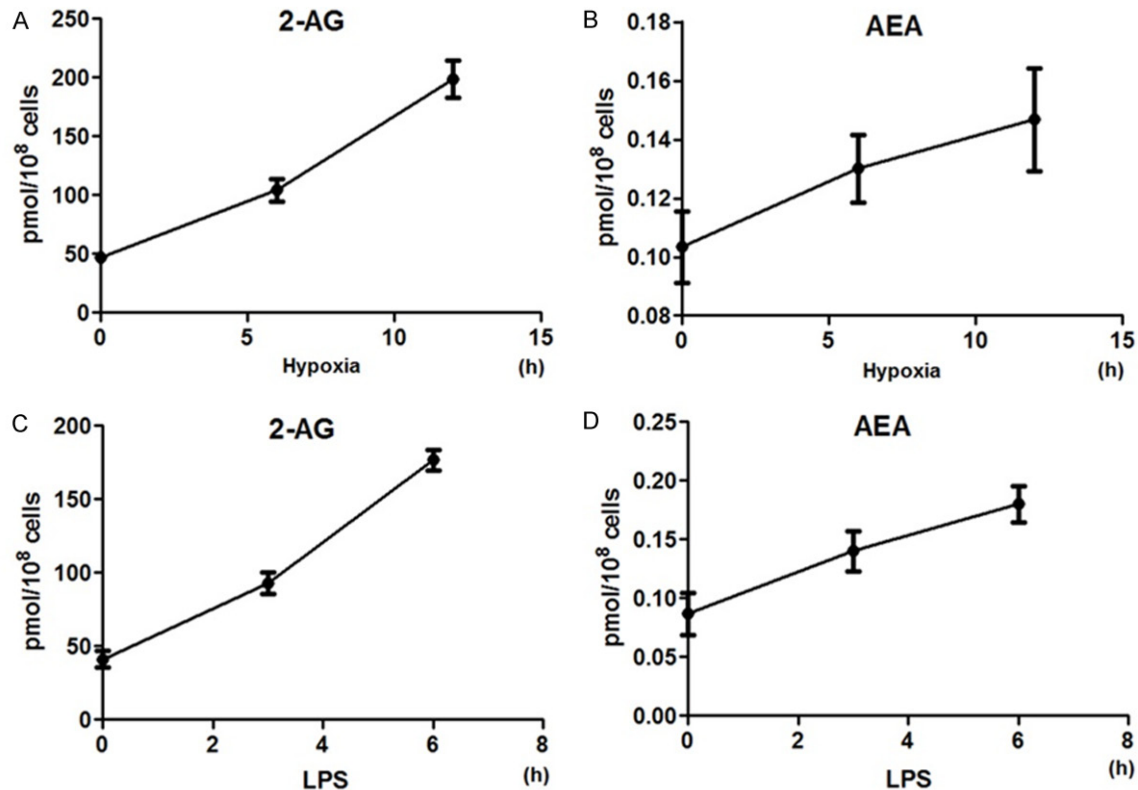

Figure 3. AEA and 2-AG levels in BV-2 cells under the condition of hypoxia or LPS. A, B. Both 2-AG and AEA levels (pmol/10^8 cells) were increased in BV-2 cells when cells were exposed to hypoxia; C, D. Similarly, when cells were treated with LPS, both 2-AG and AEA levels (pmol/10^8 cells) in BV-2 cells were increased with time increasing.

(P<0.05), suggesting the suppress effect of 2-AG on BV-2 inflammation (**Figure 4A** and **4B**). Since AEA and 2-AG can act on cannabinoid receptors, CB1 and CB2, we used antagonist of CB1 and CB2 to investigate the effects of 2-AG interacted receptors on BV-2 cell inflammations. Compared to the controls, IKBα level in BV-2 cells was increased by the antagonist application of SR144528 and SR141716A, suggesting that antagonist of CB1 or CB2 could suppress the IKBα deposition (**Figure 4C** and **4D**). Additionally, BV-2 cells were treated with both CB1 or CB2 antagonist, as well as 2-AG and hypoxia. The results showed that when cell inflammation was inhibited by 2-AG under hypoxia, IKBα level was significantly increased compare with the control (P<0.01), suggesting that CB1 or CB2 antagonist application could reverse the inhibit effects of 2-AG on BV-2 inflammation (**Figure 4E**).

Effects of activated BV-2 cells on brain neurons

To verify the inflammations in activated BV-2 cells has certain damage on brain neurons, the activated BV-2 culture which was induced with LPS at 3 h was added into the brain neurons. Consequently, the cell proliferation and apoptosis were analyzed respectively (**Figure 5**). The results showed cell viability of brain neurons was significantly inhibited with the addition of activated BV-2 cell culture (P<0.05), but caspase-3 was significantly increased compared with the control group (P<0.05), suggesting the activated BV-2 cells may bring damage to brain neurons through inhibiting neuron cell viability and inducing cell apoptosis.

Effects of endogenous cannabinoids on IMI

To investigate the potential effects of endogenous cannabinoids on IMI, inflammatory cytokine expression, and cell apoptosis were analyzed (**Figure 6**). When the rabbit IMI model was exposed to hypoxia, TNFα level was significantly increased compared to the control, but this effect was reversed by the addition of 2-AG (P<0.05), implying that endogenous cannabinoids could suppress the inflammatory reaction (**Figure 6A**). Besides, TUNEL analysis

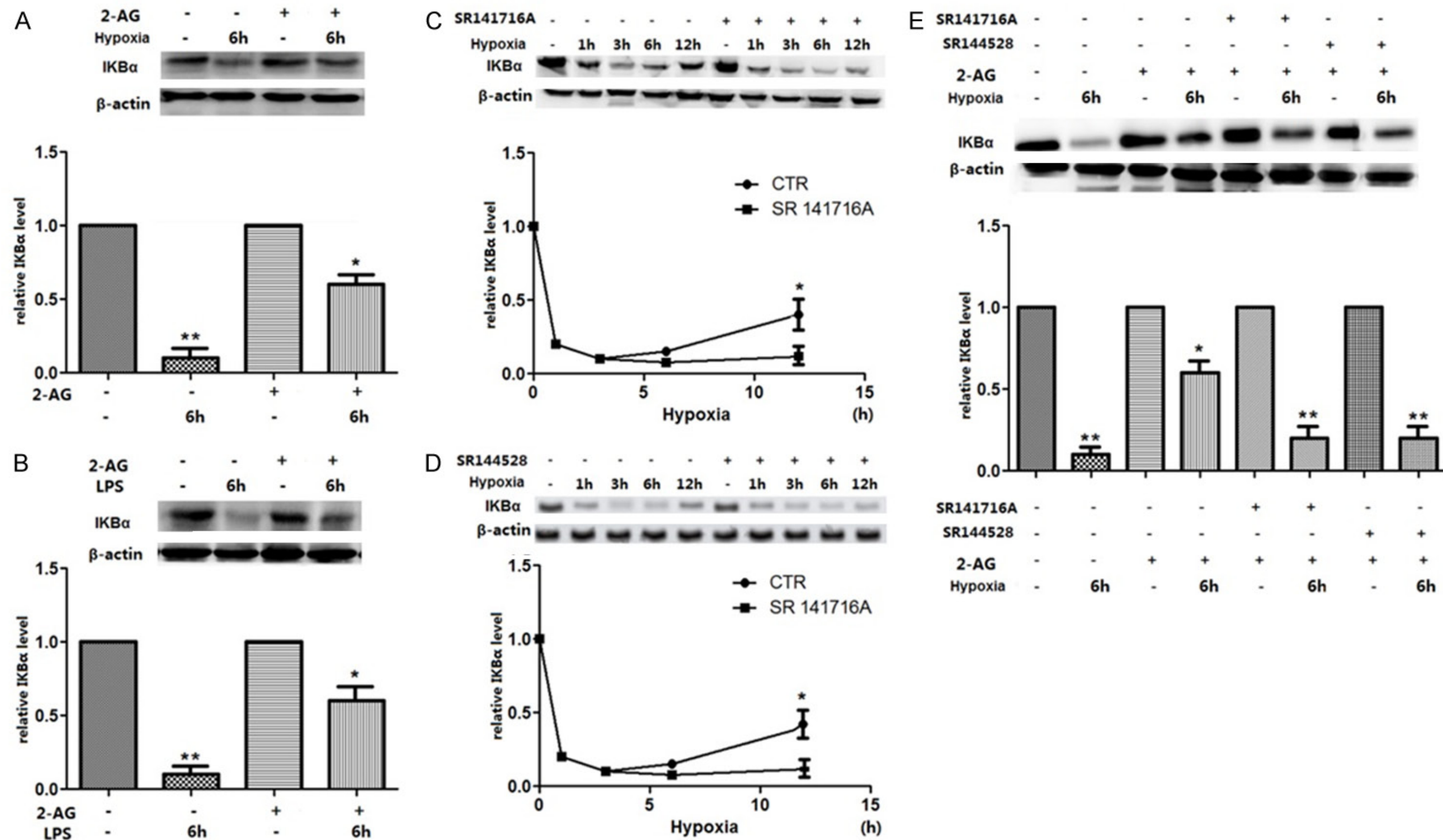

Figure 4. Correlation between the activated ECS and inflammations in BV-2 cells. A. When BV-2 cells were exposed to hypoxia, the relative IKBα level was significantly decreased, but this tendency was reversed by 2-AG application, suggesting 2-AG played certain inhibit roles on inflammations; B. Similar effects of 2-AG on inflammations in LPS treated BV-2 cells were observed, indicating the inhibit role of 2-AG on inflammations; C. When BV-2 cells inflammation was inhibited by 2-AG application, this effect was reversed by antagonist SR141716A application; D. Similar effect of antagonist SR144528 application on BV-2 cell inflammations was observed under hypoxia condition; E. When BV-2 cells were treated either with antagonist SR141716A or with antagonist SR144528, inflammatory IKBα level was significantly decreased compared to cells treated only with 2-AG under hypoxia condition. *: P<0.05, **: P<0.01 compared to the control.

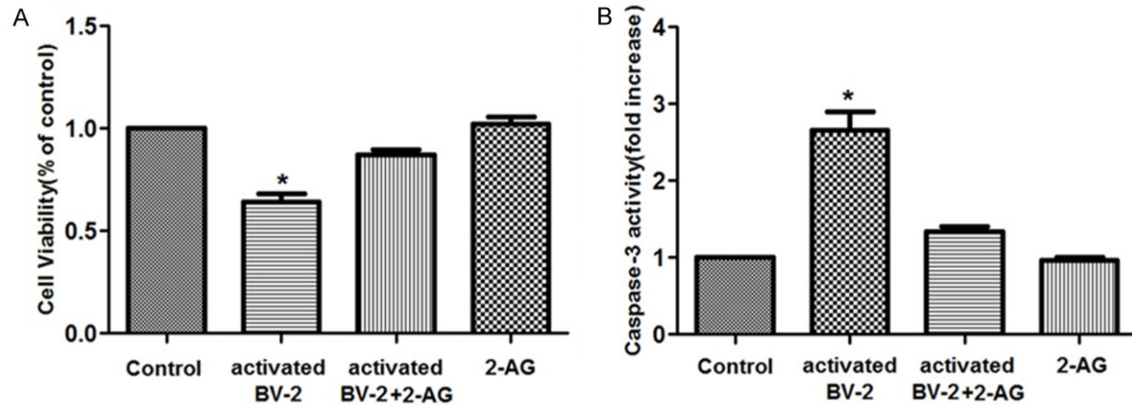

Figure 5. Influence of activated BV-2 cells on neuron cell viability and apoptosis. A. Neurons cell viability was significantly decreased when treated with activated BV-2 cells, which was induced by LPS for 3 h, however, this inhibit role of cell viability was reversed by 2-AG application; B. Caspase-3 level was significantly increased when cells were treated with the activated BV-2 cells (induced by LPS for 3 h), but this influence was reversed by 2-AG application. Results indicated that inflammations (activated BV-2 cells) would inhibit neuron cell viability but induce cell apoptosis. *: P<0.05, compared to the control.

showed that the percentage of cell apoptosis was significantly increased with hypoxia treatment, but it was decreased with 2-AG addition (**Figure 6B** and **6C**), suggesting the protective role of endogenous cannabinoids on neuron damage by reducing cell apoptosis.

Discussion

Accumulating evidence has performed the significant roles of ECS on cerebral diseases, both in central nervous system and peripheral processes [12, 13, 19]. Microglia-mediated inflammation has become one of the most pivotal mechanisms for brain diseases including IMI [7, 8]. However, few reports presented the effects of ECS on protecting brain diseases under the body self-resisting inflammations under physiological condition. In the present study, we detected the inflammation activation in constructed rabbit IMI model brain, and measured the ECS levels under this condition. Consequently, the damaged effects of inflammation, as well as the protective effects of ECS on neurons were analyzed respectively.

NF-κB activation is the central link for inflammation among the complicate cytokine network during inflammatory reaction [19], and it is positively correlated with inflammatory cytokines secretion including TNFα and IL-1β, which means the highly secreted TNFα and IL-1β will promote NF-κB activation [17]. Our results showed that TNFα and IL-1β levels in IMI model rabbit blood was significantly increased compared to the normal group (**Figure 1**). Since the brain inflammatory reaction is mediated by microglia, including TNFα and IL-1β self-activation, as well as NF-κB activation [20, 21], and the major function of IKBα is interfering the intracellular combination between NF-κB and DNA [18]. Thus, inflammatory cytokine secretion in brain-isolated microglia cells was analyzed, and results showed that IKBα level in both brain tissue and brain-isolated microglia cells was significantly decreased (**Figure 2A** and **2B**), suggesting the IMI model was successfully constructed. Furthermore, IKBα level in BV-2 cells was also detected for the verification study in vitro, and hypoxia would significantly decreased IKBα level in vitro (**Figure 2C**), suggesting that hypoxia leading to the activation of microglia-mediated inflammations during IMI in rabbit.

Accordingly, we further investigated the damages of microglia-mediated inflammations on neuron during IMI in vitro (**Figure 5**). BV-2 cells were treated with LPS for 3 h, and cell cultures were added into neurons. Won-Ki et al proved that BV-2 cells can be activated by LPS through up-regulating TNFα mRNA and protein level [22]. Inflammatory cytokines including TNFα presented toxicity effects on cerebral cell viability and apoptosis [23, 24]. Caspase-3, the symbol for cell apoptosis, was highly expressed

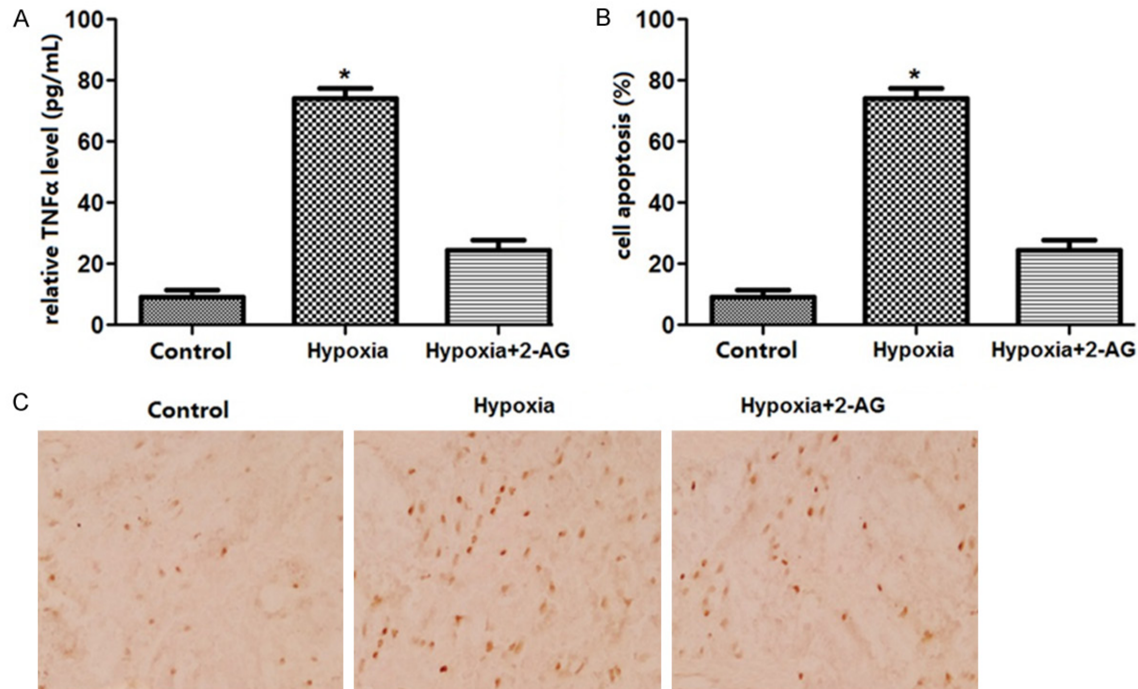

Figure 6. Protective effects of ECS on neurons under inflammations condition. A. Inflammatory reactions in neurons was induced under hypoxia condition in vitro, but was suppressed by 2-AG application; B, C. Percentage of apoptotic neurons was significantly increased compared to the control, but this effect was suppressed by 2-AG application, suggesting that 2-AG treatment would protect neurons from inflammatory damage through suppressing apoptosis. *: $P<0.05$, compared to the control.

in cerebral tissue during ischemia [25]. In this study, cell viability was inhibited while apoptosis was induced by activated BV-2 cells application (LPS-induced), indicating that the activated BV-2 cells (LPS-induced) performed harmful damage on neurons growth via activating inflammations.

On the other side, 2-AG and AEA levels in BV-2 cells under hypoxia or LPS treatment condition were measured respectively to assess the activation of ECS in microglia cells. Varga et al demonstrated that LPS can promote AEA and 2-AG production via activating microglia cells in platelets, and then leading to the decline of blood pressure [26]. In agreement with previous evidence [26-28], our data showed that both AEA and 2-AG levels in hypoxia or LPS treated BV-2 cells were increased with time increasing (**Figure 3**), which preliminary suggested ECS was activated in microglia BV-2 cells during inflammations. Consequently, the influence of ECS on microglia BV-2-mediated inflammation during IMI was further investigated. Interestingly, when hypoxia- or LPS-induced BV-2 cells were treated with 2-AG, the symbol for ECS, IKBα secretion was increased compared to the control group (**Figure 4A** and **4B**), implying the anti-inflammatory role of 2-AG in BV-2 cells. It has been said that SR141716A and SR144528 are the accordingly antagonists for CB1 and CB2, the receptors for ECS, and can be acted by 2-AG [12, 29]. Anti-inflammatory effect of 2-AG in depressing blood pressure can be antagonized by SR141716A in platelets [26]. Similar results of SR141716A on 2-AG in brain injury was reported by Panikashvili et al [27]. Based on our results (**Figure 4C-E**), we speculated that the activated ECS played certain anti-inflammatory effects on BV-2 cells during inflammatory reaction in IMI.

In consequence, we further investigated the influences of ECS on protecting neuron from damaged that induced by microglia-mediated inflammations. Activation of microglia and astrocytes in the initial injury plays pivotal roles for neuronal damage [30]. Our results showed that 2-AG application alleviated the inhibit effect of activated BV-2 (LPS-induced) on neu-

rons cell viability and apoptosis (caspase-3 level change) (**Figure 5**). Besides, 2-AG application remitted the inflammations and apoptosis in BV-2 cells under hypoxia condition (**Figure 6**), suggesting the protective role of ECS on neuron.

To sum up, the data presented in this study suggests that the activated ECS may protect neurons from being injured caused by ischemia through suppressing the excess microglia-mediated inflammation. Our study may provide theoretical basis for the anti-inflammatory drug exploration for IMI in clinical. There may be possibility for ECS on protecting neuron damage caused by IMI, therefore, further experiments are still needed to explore the deep mechanisms of ECS on protecting IMI.

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