

Epoxyeicosatrienoic acid ameliorates cerebral ischemia-reperfusion injury by inhibiting inflammatory factors and pannexin-1

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Abstract. Epoxyeicosatrienoic acid (EET) has wide applications due to the unique biological effects of anti-hyperlipidemia, inhibition of platelet aggregation, anti-inflammation, anti-cancer, anti-lipid oxidation and the promotion of brain tissue development. The present study investigated whether EET ameliorates cerebral ischemia-reperfusion injury (CIRI) by inhibiting inflammatory factors and pannexin. Specific pathogen-free 7-week-old male Sprague-Dawley rats were randomly divided into three groups: Sham, CIRI and EET. Neurological deficit scores, cerebral infarct volume and cerebral edema were assessed in CIRI rats. Enzyme-linked immunosorbent assays were performed to detect tumor necrosis factor- α , interleukin-6, nuclear factor- κ B and inducible nitric oxide synthase (iNOS) levels, and western blot analysis was performed also used to assess cleaved caspase-3, phospholipase A2 (PLA2), cyclooxygenase-2 and prostaglandin E2 (PGE2) protein expression levels. EET ameliorated cerebral injury and CIRI-induced cleaved caspase-3 protein expression levels in rats. EET additionally suppressed CIRI-induced inflammation reactions and iNOS protein expression in rats. Furthermore, the protein expression levels of PLA2, PGE2 and pannexin-1 in CIRI rats were inhibited by treatment with EET. These results indicated that EET reduces CIRI by inhibiting inflammation and levels of cleaved caspase-3, PLA2, PGE2 and pannexin-1.

Introduction

Cerebrovascular disease is the third most common cause of mortality in the world (1). It has three major characteristics: High morbidity, high disability rate and high mortality rate (1). The morbidity of cerebral ischemia-reperfusion injury (CIRI) accounts for ~70% of cerebrovascular diseases, and this trend is increasing (1). Many patients lose the ability to work and function normally, which causes heavy economic and psychological burdens for the patients, families and society (2). Therefore, it is important to prevent and control the incidence and development of CIRI.

The therapeutic principle of ischemic stroke is to restore blood perfusion in ischemia, improve the blood circulation of the brain tissue, increase blood flow and oxygen supply at ischemic penumbra, control encephaledema and to prevent and cure further complications (3,4). However, reperfusion further aggravates brain dysfunction, and structural failure results from ischemia. Brain tissue injuries cause irreversible injury of brain cells (5); this is known as ischemic CIRI. Consequently, investigations are focused on developing methods to reduce reperfusion injury, save brain cells and restore brain functions (6), potentially by detecting markers of the degree of CIRI (7). The development of novel psychotherapeutic drugs for the prevention and treatment of cerebrovascular diseases therefore has great significance.

Epoxyeicosatrienoic acid (EET; Fig. 1) was initially identified from investigations into the low incidence of cardiovascular disease in eskimos (8). Docosahexaenoic acid, eicosapentaenoic acid and other long-chain unsaturated fatty acids have the efficacy to prevent cholesterol deposition on the arterial vascular wall, and to prevent the incidence of cardiovascular disease (9). EET has been demonstrated to exert anti-inflammatory effects through nuclear factor (NF)- κ B, adipocyte protein 2 and other signaling pathways (10). The present study used a rat CIRI model to investigate the inhibition effects of EET, and the potential underlying mechanisms.

Materials and methods

Animals and experimental groups. Specific pathogen-free male Sprague-Dawley rats (age, 7 weeks; weight, 200-240 g; n=50)

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were purchased from the Center for Animal Experiments, Renmin Hospital of Wuhan University (Wuhan, China) and housed at $23\pm 2^{\circ}\text{C}$ in a 12 h light/dark cycle in $50\pm 10\%$ humidity with free access to food and water. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD, USA) and the experiments were approved by the Animal Care and Use Committee of Wuhan University. The rats were randomly divided into three groups: Sham, ($n=10$), CIRI ($n=20$) and EET ($n=20$). In the EET group, rats were administered 6.24×10^{-7} mol/l 10 ml/kg 11,12-EET via the jugular vein for 20 min. Rats in the other groups were administered with saline. All rats were intraperitoneally anesthetized with 30 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and were fixed in a supine position at $37\text{--}38^{\circ}\text{C}$. A 4/0 surgical nylon filament with a silicone-beaded tip was inserted into the right internal carotid artery via the external carotid artery to occlude the origin of the middle cerebral artery. The occlusion was released for 24 h following ischemia at 2 h.

Neurological deficit score. Rats were suspended by the tail and neurological testing was conducted independently by 3 researchers. The neurological deficit scores were as follows: 0, forelimb flexion and body twisting; 1, inability to extend the left forepaw; 2, rat circled to the left; 3, inability to move the left forepaw; 4, inability to walk.

Cerebral infarct volume and cerebral edema. To assess cerebral infarct volume, the rats were sacrificed and brain tissue samples were obtained and weighed as wet weight (WW). Subsequently, tissue samples were sectioned into 4 mm-thick pieces and stained using 2% 2,3,5-triphenyltetrazolium chloride (Amresco, LLC; Solon, OH, USA) at room temperature for 30 min. The infarct volume is expressed as the percentage of the contralateral hemisphere. Tissue samples were dried at 110°C overnight in an electric oven and weighed again to obtain the dry weight (DW). Water content (%) = $[(\text{WW}-\text{DW})/\text{WW}] \times 100\%$ was calculated as brain water content.

Enzyme-linked immunosorbent assay (ELISA). Cells were lysed using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4°C for 15–20 min, the homogenate was centrifuged for 10 min at $30,000 \times g$ at 4°C and the supernatant was collected. Protein concentrations were detected with a bicinchoninic acid (BCA) assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The levels of TNF- α (cat no. ab46070), interleukin (IL)-6 (cat no. ab100772), NF- κB (cat no. ab176647) and inducible nitric oxide synthase (iNOS; cat no. ab196266) were determined using commercial ELISA kits (Abcam, Shanghai, China).

Western blot analysis. Cells were lysed using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) at 4°C for 15–20 min, the homogenate was centrifuged for 10 min at $30,000 \times g$ at 4°C and the supernatant was collected. Protein concentrations were detected with a BCA kit. Proteins (50 μg) were separated by 10–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes

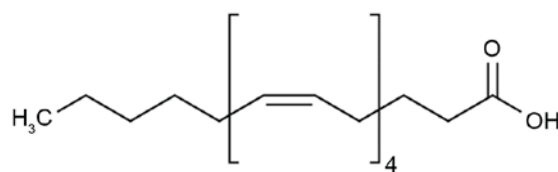


Figure 1. Constitutional formula of epoxyeicosatrienoic acid.

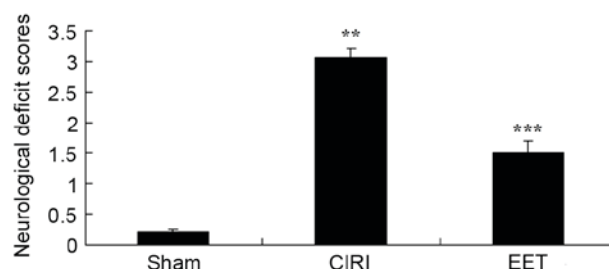


Figure 2. EET ameliorates CIRI-induced neurological deficit score. Neurological deficit scores in the sham, CIRI and EET groups. Data are expressed as the mean \pm standard deviation. **P<0.01 vs. sham; ***P<0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid.

were blocked using 5% skim milk powder in TBS containing 0.05% Tween-20 (TBST) for 1 h at 37°C and incubated overnight with the following primary antibodies at 4°C : Anti-cleaved caspase-3 (1:3,000; cat no. 9664), anti-phospholipase A2 (PLA2; 1:3,000; cat no. 2832), anti-cyclooxygenase-2 (COX-2; 1:3,000; cat no. 12282), anti-pannexin-1 (1:3,000; cat no. 91137), purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); anti-prostaglandin E2 (PGE2; 1:300; cat no. D262741) and anti- β -actin (1:3,000; cat no. D110007), obtained from Shanghai Sangong Pharmaceutical Co., Ltd. (Shanghai, China). Membranes were washed with TBST, and subsequently probed with horseradish peroxidase-labeled secondary antibodies (1:5,000; cat no. BM2006; Wuhan Boster Biological Technology, Ltd., Wuhan, China) at 37°C for 1 h. Blots were visualized by enhanced chemiluminescence using the Pierce™ Fast Western Blot kit (cat no. 35050; Thermo Fisher Scientific, Inc.) and semi-quantified using Quantity One software version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were analyzed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation of 3 independent experiments. The statistical significance of the differences between groups was assessed using a one-way analysis of variance followed by a post hoc Tukey-Kramer test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EET ameliorates CIRI-induced neurological deficit scores. Neurological deficit scores from each group are presented in Fig. 2. The neurological deficit score of the CIRI model group significantly increased compared with sham group. EET

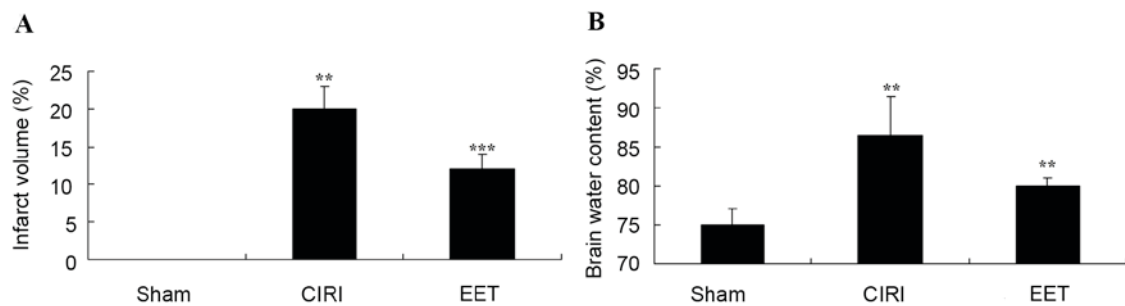


Figure 3. EET ameliorates CIRI-induced cerebral infarct volume and cerebral edema. (A) Cerebral infarct volume and (B) brain water content were assessed in the sham, CIRI and EET groups. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid.

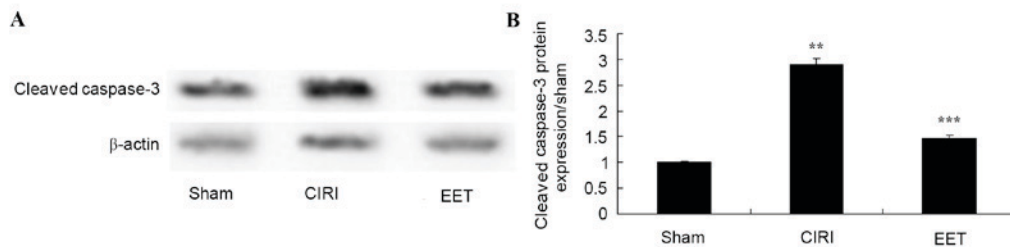


Figure 4. EET ameliorates CIRI-induced cleaved caspase-3. (A) Representative western blot images and (B) quantification of cleaved caspase-2 protein expression levels in sham, CIRI and EET-treated groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid.

treatment caused a significant decrease in the neurological deficit score, when compared with the CIRI model group.

EET ameliorates CIRI-induced cerebral infarct volume and cerebral edema. CIRI-induced cerebral infarct volume and cerebral edema (brain water content) in all groups are presented in Fig. 3. The cerebral infarct volume in the sham group was negligible, and it was significantly increased in the CIRI group in comparison. There was a significant inhibition in cerebral infarct volume in the EET group compared with the CIRI model group (Fig. 3A). Cerebral edema was increased in the CIRI group compared with the sham group; however, treatment with EET significantly reduced this effect (Fig. 3B).

EET ameliorates CIRI-induced cleaved caspase-3. As assessed by western blot analysis (Fig. 4A), cleaved caspase-3 protein expression levels in CIRI tissue homogenates was significantly increased compared with the sham group. Pretreatment with EET significantly reduced CIRI-induced cleaved caspase-3 protein expression levels (Fig. 4B).

EET ameliorates CIRI-induced inflammation. The levels of TNF- α (Fig. 5A), IL-6 (Fig. 5B) and NF- κ B (Fig. 5C) measured in CIRI group samples were significantly increased compared with the sham group; however, treatment with EET significantly ameliorated this effect.

EET ameliorates CIRI-induced iNOS activity. CIRI-induced iNOS activity was markedly increased compared with the

sham group, and EET treatment significantly attenuated this effect (Fig. 6).

EET ameliorates CIRI-induced PLA2 protein expression. PLA2 protein expression levels were determined by western blot analysis (Fig. 7A). CIRI rats exhibited significantly increased PLA2 expression levels compared with the sham group; however, EET pretreatment significantly inhibited this effect (Fig. 7B).

EET ameliorates CIRI-induced COX-2 protein expression. COX-2 protein expression levels were examined by western blot analysis (Fig. 8A). CIRI led to a significant increase in COX-2 protein expression levels compared with sham-operated rats; however, EET treatment significantly ameliorated this effect (Fig. 8B).

EET ameliorates CIRI-induced PGE2 protein expression. The protein expression levels of PGE2 was evaluated in CIRI tissue (Fig. 9A). PGE2 was highly expressed in CIRI tissue but not in sham-operated rats. In addition, this activation of PGE2 protein expression was significantly inhibited by treatment with EET (Fig. 9B).

EET ameliorates CIRI-induced pannexin-1 protein expression. Pannexin-1 protein expression levels were examined by western blot analysis (Fig. 10A). These results demonstrated that, following CIRI, pannexin-1 protein expression levels were increased compared with the sham group. Treatment with EET significantly inhibited CIRI-induced pannexin-1 protein expression in rats (Fig. 10B).

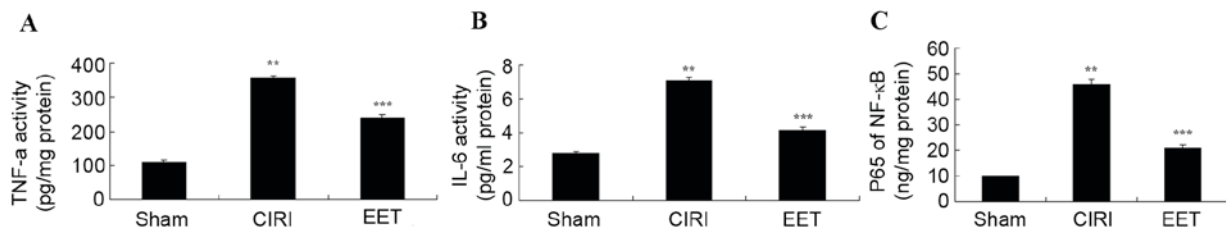


Figure 5. EET ameliorates CIRI-induced inflammation. (A) TNF- α , (B) IL-6 and (C) NF- κ B levels in the sham, CIRI and EET-treated groups, as determined by ELISA. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$ vs. sham; *** $P < 0.01$ vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid; NF- κ B, nuclear factor- κ B; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α .

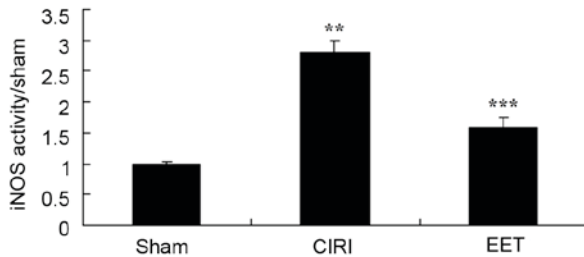


Figure 6. EET ameliorates CIRI-induced iNOS activity. iNOS activity in the sham, CIRI and EET-treated groups, as determined by enzyme-linked immunosorbent assay. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$ vs. sham; *** $P < 0.01$ vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid; iNOS, inducible nitric oxide synthase.

Discussion

CIRI refers to cellular function metabolic disorders and organizational structure damage aggravation following blood reperfusion of ischemic damage (11). Although timely blood reperfusion may rescue certain cells close to the site of infarction, reperfusion causes a greater extent of tissue damage than pure ischemia injury (11). Previous studies have indicated that CIRI is primarily associated with inflammatory reactions, excitability amino acids, intracellular friction overload, oxygen free radicals and cell apoptosis (4,12). These various factors interact during cerebral ischemia reperfusion, and interlink to form a vicious circle. It eventually leads to ischemia reperfusion injury (13). Among these factors, the inflammatory reaction is considered to be one of the most important causes of CIBI (13). TNF is a cytokine with key effects in the network of inflammation. It is considered as an initiating medium for systemic inflammatory reaction (14). It may directly lead to reduced circulation resistance, increased vasopermeability and decreased numbers of vascular endothelial cells. In addition, it may induce a cascade release of IL-1, IL-6 IL-8 and additional cytokines, which forms amplification effects on inflammatory injury (14). The present study demonstrated that EET treatment significantly ameliorated CIRI-induced neurological deficit scores, cerebral infarct volume and cerebral edema, and inhibited the expression levels of cleaved caspase-3, TNF- α , IL-6 and NF- κ B in CIRI rats. Previous studies have demonstrated that EET attenuates IL-8 production in bronchial epithelial cells (15) and inflammation in the cardiovascular system (16).

PLA2 has been suggested as an inflammatory marker closely associated with atherosclerosis (17). A combination of

PLA2 and low-density lipoproteins in the blood generates a great amount of blood transportation, lecithin and free fatty acid oxidation, promoting atherosclerosis (18). The primary pathological basis of ischemic stroke, particularly cerebral thrombosis, is atherosclerosis (19). Consequently, it is a predictive index for atherosclerosis cerebral infarction. The present study revealed that EET treatment significantly ameliorates CIRI-induced PLA2 protein expression in rats. Jiang *et al* (20) reported that the release of EETs from red blood cells may be via cytosolic PLA2.

Increased expression levels of PLA2 may induce genetic expression of COX-2 for numerous inflammation-stimulating factors, including lipopolysaccharide, IL-1, TNF, epidermal growth factor α and platelet activating factor (21), and increase levels of PGE2, prostacyclin and PGE1 at inflammatory sites. A positive association between COX-2 mRNA and PGE2 levels at inflammatory sites and severity of inflammation has been verified in numerous experiments (22). At present, PGE2 is considered as an important mediator of inflammation. It has been demonstrated that the expression of COX-2 in the brain is the highest among all visceral organs, and is primarily distributed in the hippocampus and temporal lobe cortex (23,24). This may be associated with the high density of excitatory amino acid receptors in these regions. Overexpression of COX-2 in nervous tissue may directly damage nerve cells (25). In the present study, COX-2 protein expression levels were significantly reduced by treatment with EET. Michaelis *et al* (26) indicated that EET induces COX-2 protein expression in endothelial cells.

The gap-junction connected gel networks of astrocytes have been suggested to be involved in ischemia; therefore, have attracted increasing attention (27). Pannexin, the primary protein composition for close connection of astrocytes, is involved in transmission of information within astrocyte networks via communication between gap junctions (27). Gap junction intercellular communication may disperse antioxidative, anti-apoptotic and growth factors around the ischemia area via tight junctions to alleviate ischemic injury. However, dying nerve cells in the ischemic core area may disperse excitatory amino acids, free radical products, apoptosis signals and other injurious factors to ischemia area to induce cell injury and apoptosis in the peripheral zone, further expanding the cerebral infarction volume (28,29). The present study demonstrated that EET ameliorates CIRI-induced PGE2 and pannexin-1 protein expression levels in rats. Nüsing *et al* (30) suggested that EET may affect renal tubular epithelial cells via PGE2 and COX-2.

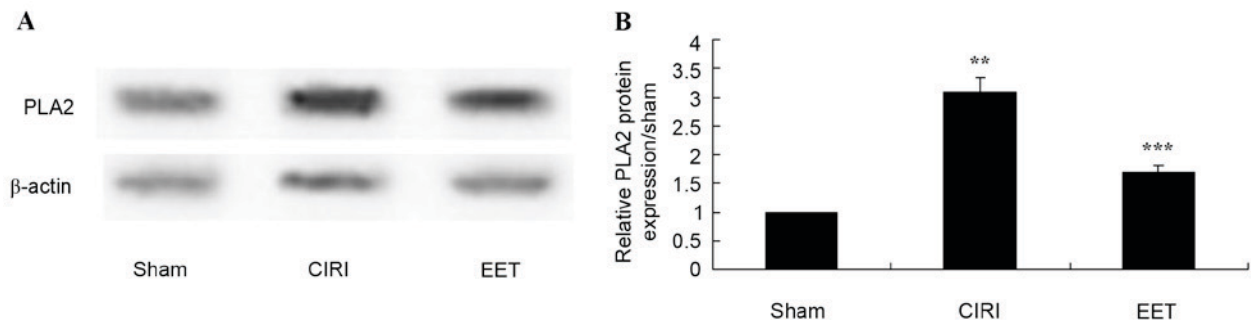


Figure 7. EET ameliorates CIRI-induced PLA2 protein expression. (A) Representative western blot images and (B) quantification of PLA2 protein expression levels in sham, CIRI and EET-treated groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid; PLA2, phospholipase A2.

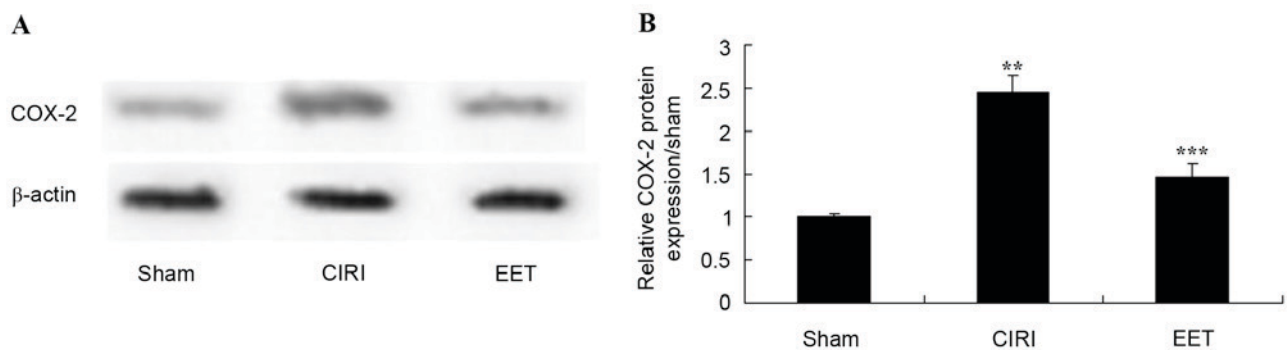


Figure 8. EET ameliorates CIRI-induced COX-2 protein expression. (A) Representative western blot images and (B) quantification of COX-2 protein expression levels in sham, CIRI and EET-treated groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid; COX-2, cyclooxygenase 2.

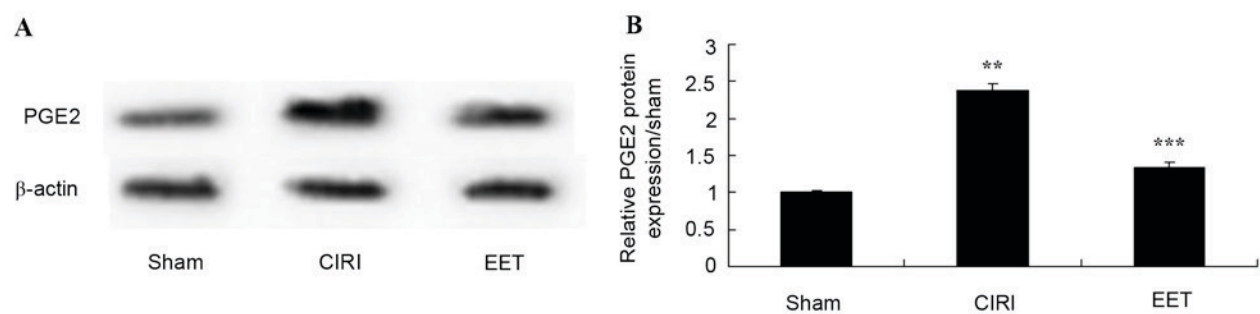


Figure 9. EET ameliorates CIRI-induced PGE2 protein expression. (A) Representative western blot images and (B) quantification of PGE2 protein expression levels in sham, CIRI and EET-treated groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid; PGE2, prostaglandin E2.

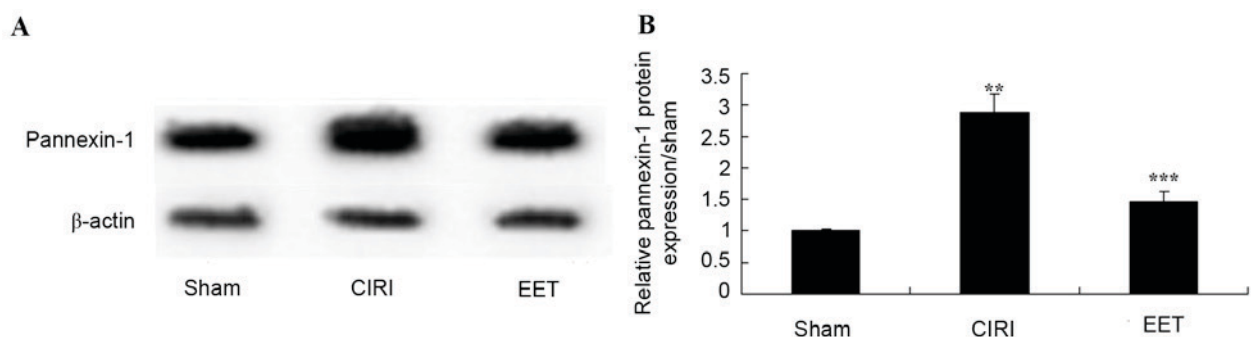


Figure 10. EET ameliorates CIRI-induced pannexin-1 protein expression. (A) Representative western blot images and (B) quantification of pannexin-1 protein expression levels in sham, CIRI and EET-treated groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ** P <0.01 vs. sham; *** P <0.01 vs. CIRI. CIRI, cerebral ischemia-reperfusion injury; EET, epoxyeicosatrienoic acid.

In conclusion, the present study demonstrated that EET inhibits CIRI-induced neurological deficit scores, cerebral infarct volume and cerebral edema, and inhibited the levels of cleaved caspase-3, TNF- α , IL-6 and NF- κ B in CIRI rats via inhibiting the activation of PLA2/COX-2/PGE2. Therefore, EET may represent a potential therapeutic agent for the prevention and treatment of CIRI.

Acknowledgements

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