Molecules and Cells

Epoxyeicosatrienoic Acid Inhibits the Apoptosis of Cerebral Microvascular Smooth Muscle Cells by Oxygen Glucose Deprivation via Targeting the JNK/c-Jun and mTOR Signaling Pathways

Youyang Qu, Yu Liu, Yanmei Zhu, Li Chen, Wei Sun*, and Yulan Zhu*

Department of Neurology, the Second Affiliated Hospital of Harbin Medical University, Harbin 150086, P. R. China
*Correspondence: ylz_yulan@outlook.com (YZ); sw191190@163.com (WS)
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As a component of the neurovascular unit, cerebral smooth muscle cells (CSMCs) are an important mediator in the development of cerebral vascular diseases such as stroke. Epoxyeicosatrienoic acids (EETs) are the products of arachidonic acid catalyzed by cytochrome P450 epoxygenase. EETs are shown to exert neuroprotective effects. In this article, the role of EET in the growth and apoptosis of CSMCs and the underlying mechanisms under oxygen glucose deprivation (OGD) conditions were addressed. The viability of CMSCs was decreased significantly in the OGD group, while different subtypes of EETs, especially 14,15-EET, could increase the viability of CSMCs under OGD conditions. RAPA (serine/threonine kinase Mammalian Target of Rapamycin), a specific mTOR inhibitor, could elevate the level of oxygen free radicals in CSMCs as well as the anti-apoptotic effects of 14,15-EET under OGD conditions. However, SP600125, a specific JNK (c-Jun N-terminal protein kinase) pathway inhibitor, could attenuate oxygen free radicals levels in CSMCs as well as the anti-apoptotic effects of 14,15-EET under OGD conditions. These results strongly suggest that EETs exert protective functions during the growth and apoptosis of CSMCs, via the JNK/c-Jun and mTOR signaling pathways in vitro. We are the first to disclose the beneficial roles and underlying mechanism of 14,15-EET in CSMC under OGD conditions.

Keywords: cerebral ischemia/reperfusion injury, epoxyeicosatrienoic acids, JNK/c-Jun pathway, mTOR pathway

INTRODUCTION

Stroke is a disorder of blood vessels, which can affect neurons and cause ischemic brain injuries. Both aberrant regulations of macro- and micro-vascular conditions occur during ischemia/reperfusion (I/R), which can aggravate the primary condition and cause secondary brain injuries such as hemorrhage and edema. The cerebral vasculature plays a central role in stroke damage because core infarction is affected by the depth and duration of ischemia (Palomares and Cipolla, 2011). The neurovascular unit not only restricts and controls the entry of blood composition into the cerebral parenchymatous tissues but also plays an important role in fulfilling the metabolism requirements established by neuronal activities (Abbott et al., 2006; Iadecola, 2004). As a component of the cerebral vasculature and an important part of the neurovascular unit, the cerebral microvasculature plays important roles in ischemic brain injuries. Lee and colleagues have reported that effective post-stroke reperfusion depends on functional cerebrovascular networks formed by small

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arteries so that parenchymal capillaries can be nourished. Cerebral capillaries originate from parenchymal arterioles and ramify exuberantly to establish the capillary microcirculation inside the brain (Lee, 1995). In addition, the intraparenchymal arterioles stem from pialarteries while nourishing and penetrating through different parts of the brain (Lee, 1995). All these arteries and downstream arterioles contain an important type of cells: vascular smooth muscle cells (Lee, 1995).

Ischemic stroke is a vascular disorder affecting neuronal function. Because ischemia affects not only neurons but also astrocytes and other glial cells that support the neurons, and the microvessels that supply oxygen and nutrients to them. Neurons and microvessels respond equally rapidly to the acute ischemic insult. Although the ability of vascular smooth muscle cell to tolerate OGD may be higher than that of nerve tissue (Mabuchi et al., 2005). During the acute phase of cerebral ischemia, vascular injury mainly refers to cerebral microvascular system, which results in increased Blood Brain Barrier permeability. Cerebral microvascular SMCs play important roles in the physiology and pathology of cerebral ischemia (Dirnagl, 2012). In brief, as a component of neurovascular unit, CSMCs are important mediators in the development of cerebral vascular diseases such as stroke. Many studies have focused on the roles of CSMCs in ischemic brain injury to investigate their functions and underlying mechanisms.

Epoxyeicosatrienoic acids (EETs) containing four regioisomers (5,6-, 8,9-, 11,12- and 14,15-EET) are produced from arachidonic acid (AA) as it is processed by cytochrome P450 epoxygenase. EETs participate in multiple intracellular processes, such as anti-inflammatory and vasodilating responses, while at the same time could adjust blood flow in the brain to fulfill metabolism requirements and maintain neural activities (Koehler et al., 2009; Imig, 2010; Sudhahar et al., 2010). EETs also modulate angiogenesis via a nitric-oxidedependent mechanism as well as via activation of PI3-kinase and MAPK pathways (Wang et al., 2003) and increase K^+ currents in cerebral arteriolar smooth muscle cells(Gebremedhin et al., 1992; Medhora et al., 2001).

Our previous study has shown that the beneficial role of EETs in cerebral I/R injury involves the PI3K/Akt pathway as well as ATP-sensitive potassium (KATP) channels (Qu et al., 2015). Nevertheless, the cytoprotective functions of EETs have not been completely investigated in CSMCs.

The JNK (c-Jun N-terminal protein kinase) signaling pathway is associated with neuron apoptosis due to global or focal ischemia (Selimi et al., 2000). Previous studies suggested that activated JNK pathway is actively involved in brain I/R injury (Drobny and Kurca, 2000; Heil and Schaper, 2004). Hence, JNK has been the key in the treatment to prevent cerebral ischemia events. Other studies showed that EETs on the one hand promote pulmonary artery endothelial cells angiogenesis, and on the other hand prevent cells to undergo apoptosis through the JNK/c-Jun pathway (Ma et al., 2012). The interactions between the JNK pathway and EETs in terms of brain I/R injury currently remain largely unknown.

Mammalian target of rapamycin (mTOR) is a critical moderator during protein synthesis, while participates in a number of biological processes, including cell proliferation and growth. Some studies have suggested that rapamycin exerts a protective role during I/R, whereas other researchers have demonstrated that mTOR participates in I/R preconditioning (Brakemeier et al., 2003; Tong et al., 2000). It remains enigmatic whether the protective functions of EETs could be regulated by the mTOR pathway during cerebral I/R injury.

The aim of this study was to investigate the functions of EET in CSMC and elucidate the intrinsic molecular mechanism. It was confirmed that EET functioned by regulating both JNK/c-Jun and mTOR pathways. The results were obtained based on a cellular model of OGD.

MATERIALS AND METHODS

Materials

14,15-EET, 11,12-EET and 8,9-EET compounds were obtained from Cayman Chemical Corporation (USA). JNK antibodies and c-Jun antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-mTOR rabbit polyclonal antibodies and anti-phospho mTOR rabbit polyclonal antibodies were purchased from Cell Signaling Technology (USA). SP600125, which is a specific JNK inhibitor, and rapamycin (RAPA), which is a specific MTOR inhibitor, were purchased from Sigma (USA).

Male adult Sprague-Dawley (SD) rats of 250-280 g in weight were used in this study. The protocol complied with the ethical regulations at Harbin Medical University.

Tissue culture

Primary CSMCs were obtained from 10-day old SD rats following a previously published method (Fang et al., 2002). The collected cells were transferred into a DMEM medium containing 1% penicillin/streptomycin, 1% glutamine, and 10% fetal bovine serum. The cell suspension was centrifuged for 10 min to obtain cell pellets. Cell culture was maintained in a humidified chamber at 37℃ with 5% CO_2 and 85% humidity for 3 to 5 days. The proportion of CSMC in the culture was observed using an anti-smooth-muscle-actin monoclonal antibody (Boehringer Mannheim). The results of immunostaining are shown in Fig. 1A.

OGD and reoxygenation

Several published articles have used OGD of vascular smooth muscle cell for cerebral vascular research (Meng and Yu, 2011; Qu et al., 2015; Zhang et al., 2016).The cells were incubated under normal conditions for 24 h in order to achieve 80-90% confluence. Before OGD, the cells were treated with compounds as indicated in experiments for 30 min. For the experimental groups, the culture dishes were kept at 37℃ in an anaerobic and humidified chamber containing a gas mixture of 1% O_2, 3% CO_2 and 92% N_2. The cells were subjected to four-hour incubation in a DMEM/F12 medium containing no glucose and serum. At the end of 4 h hypoxia exposure, reoxygenation was carried out for 24 h under normal conditions. At the mean time, control cells were kept in a HBSS buffer containing 15 mM glucose and the oxygen level in the incubator was normal.

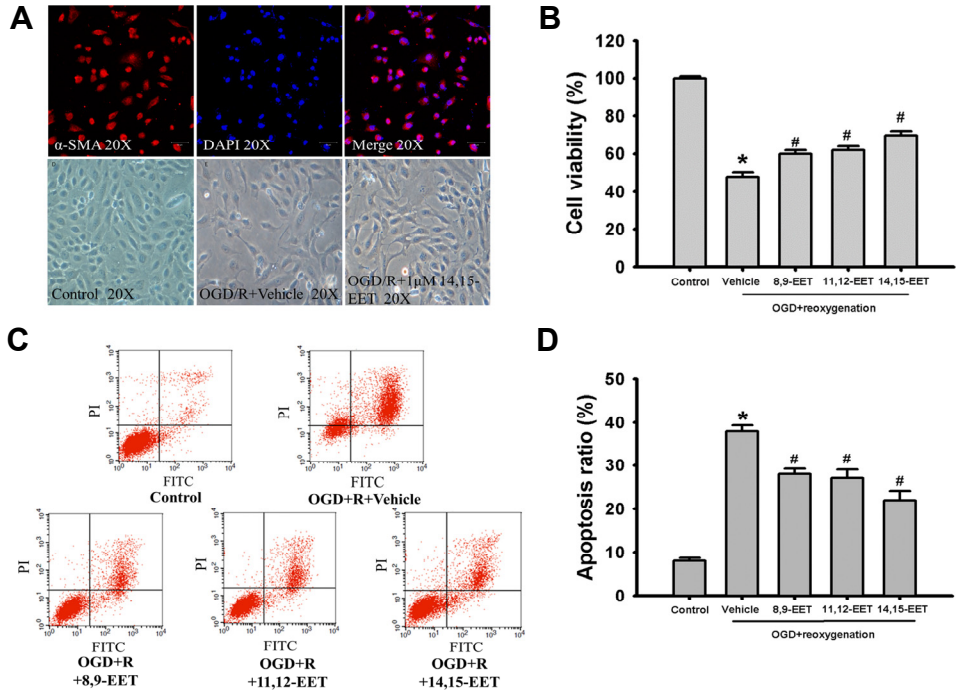

Fig. 1. EETs promote the survival of CSMCs treated by oxygen glucose deprivation and reoxygenation (we carried out reoxygenation for twenty-four hours following four-hour exposure to hypoxia). (A) Cells demonstrated a uniform pattern of immunostaining for DAPI and α-smooth muscle cell actin. The cells were double positive for α-smooth muscle cell actin and DAPI. (B) Viability of CSMC. The 1 μM each of 8,9-EET, 11,12-EET or 14,15-EET was added onto CSMC at 30 min before exposing to oxygen glucose deprivation. After treatments by oxygen glucose deprivation and reoxygenation, the cell viability was determined by the MTT assay. (C) Representative flow cytometry results demonstrated PI and annexin V-FITC staining. (D) Results from flow cytometry. The apoptotic ratio was calculated as the ratio of the number of PI-negative and annexin V-positive cells and the total number of analyzed cells. The data were presented as means ± standard deviation; $n = 3$. $^{\#}P < 0.05$, compared to OGD+ vehicle; $*P < 0.05$, compared to controls.

Flow cytometry

The rate of apoptosis was measured with flow cytometry, using a previously described protocol (Kumar et al., 2012). After the respective treatments, cells were harvested by centrifugation at 1000 rpm for 5 min, followed by re-suspending at the density of 1×10^6 cells/ml. A total of 500 μl cells were incubated with propidium iodide (PI; 10 μl at 20 μg/ml) and fluorescein isothiocyanate (FITC)-Annexin V (5 μl) for 10 min at room temperature and in the dark. At the end of 10 min incubation, the cells were analyzed immediately with flow cytometry. The FACSCalibur cell sorter (Beckman Coulter, America; BD FACSVantage SE) was utilized to perform flow cytometry (emission wavelength, 530 nm; excitation wavelength, 488 nm). Each cell sample was analyzed in triplicate, while statistical analysis was based on three independent experiments.

Cell viability

Subsequently, 1 μM of 8,9-EET, 11,12-EET or 14,15-EET was added into the medium, followed by 30 min of incubation prior to the OGD treatment. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to detect the survival rate of CSMC. Following OGD and reoxygenation treatment of the cells for twenty four hours, 10 μl of MTT solution (0.5 mg/ml) was added into each well. And then they were subjected to incubation at 37℃ for four hours. The values of optical density (OD) at 570 nm were measured by a microplate spectrophotometer. Cells undergoing no treatment were used as controls, while the medium containing MTT solution but no cells was employed as the background reference. The 8 samples from each group were measured at least three times for MTT assay, The data regarding cell viability were presented relative to the viability of untreated controls.

Western blot

To study *in vitro* effects of EETs, 1 μM 14,15-EET were put into the medium and incubated for half an hour. CSMC of rats were collected into a buffer containing 1% TritonX-100, 50 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl and 2 mM PMSF. Cells were rinsed with PBS (cold phosphate buffered saline) before incubating in a lysis buffer for half an hour on ice. In order to measure the *in vitro* effect of 14,15-EET on JNK/c-jun and mTOR pathways, the cells were centrifuged for 15 min at 13,000 rpm and 4℃, and the supernatant was used for experiments. Subsequently, 15 μg of proteins underwent 12% SDS-PAGE and were transferred onto nitrocellulose membranes, which were then blocked in 5% bovine

serum albumin (BSA). Western blot experiments were performed using antibodies against p-mTOR, mTOR, JNK, c-JUN, Bcl-2, and Bax. Immunoreactive bands were used for densitometric analysis, in combination with an enhanced chemiluminescence detection system (Amersham, USA).

Hoechst Staining

The cells grew in a six-well plate until reaching 80% confluency. Subsequently, the cells were treated with 14,15-EET and underwent OGD and reoxygenation (OGD+R) treatment, followed by 30 min staining with 1 μl of Hoechst 33342 (5 mg/ml) which was diluted in 1 ml of basal medium. The cells were rinsed twice in cold phosphate buffered saline (Sigma, USA), and observed using fluorescence microscopy at the excitation wavelength of 350 nm and emission wavelength of 460 nm.

Caspase-3 activity

Caspase-3 activity was determined by examining the cleavage of its substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). The resulting absorbance was detected at 405 nm using a commercial kit. Samples were prepared according to the western blot method described above. During the assay, cell supernatant containing 50 μg of total protein was added into a buffer solution containing 2 mM Ac-DEVD-pNA and was subjected to incubation at 37℃ for four hours. After that, the absorbance value of yellow pNA was acquired at 405 nm. Based on the total protein, Caspase-3 activity was standardized and was expressed as the fold change in caspase-3 baseline activity of the control cells, which were subjected to culture in DMEM containing 10% cold phosphate buffered saline.

ROS production and markers for oxidative stress

Reactive oxygen species (ROS) production in CSMCs was investigated using the fluorescent probe 29,79-dichlorodihydrofluorescin (DCF) diacetate, following a previously described method (Robertson et al., 2000). Briefly, CSMCs were cultured at 37℃ for 45 min in 50 mmol/l DCF diacetate. We detected the fluorescence of DCF using a multi-well plate reader.

Following the instructions, SOD (superoxide dismutase, an oxidative stress marker) and the content of MDA (malondialdehyde) in CSMC were detected using an assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analysis

All data are described as mean ± SD. *t*-test was used to analyze difference in terms of infarct size in two groups, and the difference among multiple groups was analyzed using one-way analysis of variance (ANOVA) and the subsequent Student-Newman-Keuls test. A value of $P < 0.05$ indicated statistical significance.

RESULTS

EETs protect CSMCs against cellular damage as well as OGD and reoxygenation induced cell apoptosis

To study the protective roles of EETs in rat CSMCs, we first analyzed cell viability using the MTT assays. Cell viability was decreased compared to the controls after treatments of OGD and reoxygenation (Fig. 1B). Subsequently, CSMCs were treated with 1 μM 8,9-EET, 11,12-EET or 14,15-EET for 30 min prior to OGD exposure. The IC50 of 8,9-EET, 11,12-EET and 14,15-EET were 1.28 μM, 1.54 μM and 1.80 μM respectively. Compared with cells treated with 14,15-EETs, OGD decreased cell viability to 52% of the control, whereas the cell survival rate was around 80% of the control. From the flow cytometry results (Figs. 1C and 1D), pretreatment with 1 μM 8,9-EET, 14,15-EET or 11,12-EET decreased cell apoptosis ratios as compared with the cells treated by vehicles and subjected to OGD and reoxygenation. The results suggested that EETs protect CSMC against OGD and reoxygenation-induced apoptosis. Among the three isoforms of EETs, 14,15-EET showed the strongest effects on CSMCs.

14,15-EET enhances the phosphorylation of mTOR and attenuates c-jun and JNK activation in CSMCs undergoing OGD

Activated PI3K/Akt/MTOR signaling pathway potentiates survival of cells while suppresses apoptosis after cerebral ischemia (Huang et al., 2014). The levels of phosphorylated and total mTOR in the primary CSMC culture pretreated with 1 μM 14,15-EET for half an hour before exposing to OGD were analyzed. As demonstrated in Fig. 2A, OGD induced a significant decrease in the ratio of p-mTOR/mTOR in CSMCs, as compared with that in the controls. Pretreatment with 14,15-EET prevented OGD-induced down-regulation in the level of mTOR, suggesting that in CSMCs, mTOR phosphorylation may participate in 14,15-EET-induced protection against OGD.

To ascertain whether the JNK/c-Jun pathway is affected by 14,15-EET in CSMC, the protein levels of JNK and c-jun were measured. As shown in Fig. 2B and 2C, treatment of CSMC by OGD and reoxygenation up-regulated the levels of p-c-jun and p-JNK among the OGD and reoxygenation treatment group (OGD+R). The application of 1 μM 14,15-EET significantly inhibited p-c-jun and p-JNK expression as compared with that in the group of OGD+R+vehicle treatment group. These results suggest the potential regulatory role of JNK/c-jun and mTOR in the anti-apoptotic effect of EETs.

JNK/c-Jun and mTOR pathways participate in the anti-apoptotic functions of 14,15-EET in CSMC undergoing OGD

RAPA, a specific mTOR inhibitor, and SP600125, a specific JNK inhibitor, were employed to investigate whether the disturbance of mTOR and JNK/c-jun pathways affected the anti-apoptotic functions of 14,15-EET in CSMCs undergoing OGD. Representative images of nuclei which were stained by Hoechst 33342 are demonstrated in Fig. 3. There was obvious condensation of chromatin in OGD-treated cells but such phenomenon was absent in control cells; notably, the degree of chromatin condensation was reduced in cells treated with 14,15-EET after OGD and reoxygenation.

DNA fragmentation was prevented by 14,15-EET, as suggested by the decreased degree of nuclear fragmentation in 14,15-EET-pretreated cells. However, in the presence of RAPA,

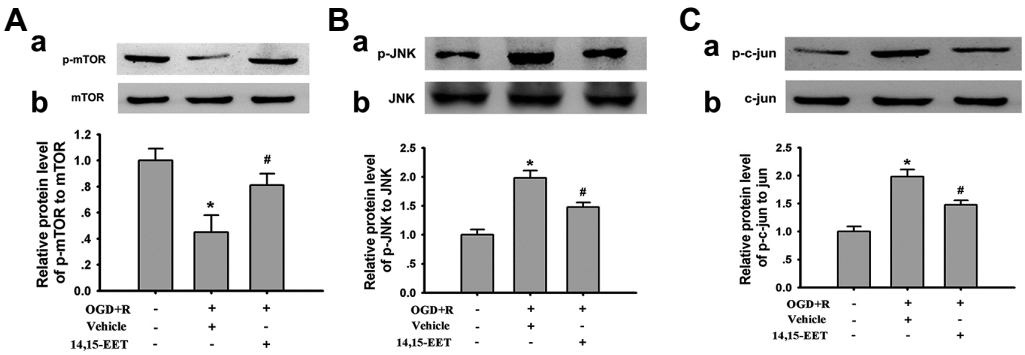

Fig. 2. The phosphorylation of mTOR, JNK and c-Jun in CSMC exposed to oxygen glucose deprivation and reoxygenation was regulated by 14,15-EET. 14,15-EET (1 μM) was added onto CSMC at half an hour prior to exposing to oxygen glucose deprivation. The levels of p-mTOR and mTOR, p-JNK and JNK, p-c-jun and c-jun expression were detected by western blot after the treatment of oxygen glucose deprivation and reoxygenation. (A) Representative photos and quantitative analysis results for the densitometric ratio of p-mTOR and mTOR protein expression in CSMC under different conditions. (B) Representative photos and quantitative analysis results for the densitometric ratio of p-JNK and JNK protein expression in CSMC under different conditions. (C) Representative photos and quantitative analysis results for the densitometric ratio of p-c-jun and c-jun protein expression in CSMC under different conditions. The data were presented as means ± standard deviation; $n = 3$. $^{\#}P < 0.05$, compared to OGD+vehicle; $*P < 0.05$, compared to controls.

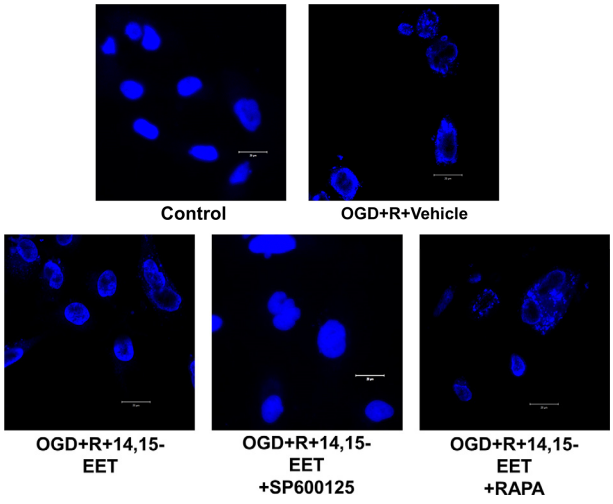

Fig. 3. 14,15-EET blocked the fragmentation of nuclei by oxygen glucose deprivation and reoxygenation. The effect was mediated by the JNK inhibitor (SP600125) and mTOR inhibitor (RAPA). The cells were treated with 1 μM 14,15-EET, 10 nM RAPA or 5 μM SP600125 for 30 min before exposing to oxygen glucose deprivation. Reoxygenation was carried out for 24 h following 4 h exposure to hypoxia. Hoechst reagent was employed for nuclear staining, which was imaged.

the protective effect of 14,15-EET was reduced. Notably, in the presence of SP600125, the protective effect of 14,15-EET was enhanced.

In CSMCs undergoing OGD, mTOR and JNK/c-Jun pathways participate in the regulation of apoptotic proteins expression and caspase-3 activity mediated by 14,15-EET

We next investigated whether regulated the expression of apoptotic proteins in CSMC under OGD conditions. Relative to that in the OGD group, 14,15-EET could upregulate the expression of Bcl-2, an anti-apoptotic protein, and down-regulate Bax expression in CSMCs under OGD conditions (Figs. 4A and 4B). However, these effects were largely eliminated by blocking the mTOR pathway. In the presence of the JNK inhibitor, Bcl-2 expression was even up-regulated while the Bax level was suppressed relative to that in the 14,15-EET+OGD+R group. Caspase-3 activity was increased in CSMC after treatment with OGD and reoxygenation. Nevertheless, caspase-3 activity was significantly decreased when cells were pretreated with 14,15-EET (Fig. 4C). RAPA upregulated caspase-3 activity as compared to that in the 14,15-EET+OGD+R group. However, in the presence of SP600125, caspase-3 activity was inhibited as compared to that in the 14,15-EET+OGD+R group. These findings indicated that the anti-apoptotic effect of 14,15-EET may involve the balance among the activities of Bax, Bcl-2 and caspase-3, which was mediated by both the mTOR and JNK/c-jun pathways.

14,15-EET affects ROS levels, SOD activity and MDA content in CSMC undergoing OGD

To evaluate the level of stress in CSMC of the OGD +R group after treating the cells with 14,15-EET, the activity of the CSMCs was determined by measuring the total levels of SOD and ROS, as well as the MDA content (Fig. 5). The SOD activity was inhibited, while the MDA content and ROS levels were increased in the OGD+R group as compared to the values in the control group. When pretreating CSMC with 1 μM of 14,15-EET for 30 min prior to OGD and reoxygenation exposure, the SOD activity in CSMC was increased, while the MDA content and ROS levels were suppressed as compared with the values in the OGD+R group. The results showed that 14,15-EET protected CSMC undergoing OGD, by regulating the release of oxygen free radicals.

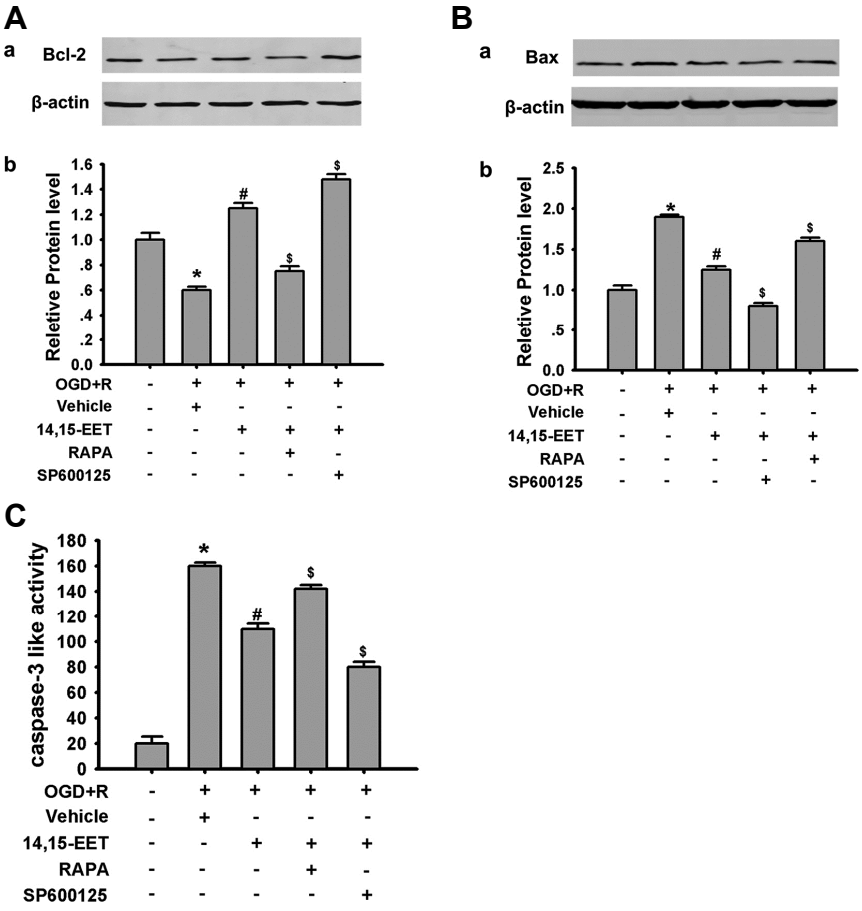

Fig. 4. 14,15-EET affected the expression of Bcl-2 and Bax, and activity of caspase-3 in CSMC. The cells were treated with 1 μM 14,15-EET,10 nM RAPA or 5 μM SP600125 for 30 min before exposing to oxygen glucose deprivation. Reoxygenation was carried out for 24 h following 4-h exposure to hypoxia. (A) Representative photos and quantitative analysis results for the densitometric ratio of Bcl-2 expression in CSMC under different conditions. (B) Representative photos and quantitative analysis results for the densitometric ratio of Bax expression in CSMCs under different conditions. (C) Caspase-3 activity in CSMC under different conditions. The data were presented as means ± standard deviation; $n = 3$. $*P < 0.05$, compared to controls; $^{\$}P < 0.05$, compared to OGD+14,15-EET; $^{\#}P < 0.05$, compared to OGD+vehicle.

To investigate whether the JNK/c-jun and mTOR pathways were involved in mediating the protective functions of 14,15-EET during OGD-induced injury, RAPA and SP600125 were used to block the mTOR and JNK/c-jun pathways, respectively. The SOD activity was decreased while the MDA content and ROS levels were elevated after blocking the mTOR pathway compared with the values in the 14,15-EET+OGD+R group. However, when the JNK/c-jun pathway was blocked, the SOD activity was increased while the MDA content and ROS levels were decreased compared with the values in the 14,15-EET+OGD+R group, in contrast to the effect observed during mTOR inhibitor treatment.

DISCUSSION

Here we demonstrated for the first time that different subtypes of EETs exerted protective functions during the apoptosis and proliferation of CSMCs under OGD conditions. In addition, we found that 14,15-EET suppressed OGD-induced apoptosis in CSMCs, potentially by activating the mTOR pathway and inhibiting the JNK pathway. Furthermore, we evaluated the effects of 14,15-EET on the levels of apoptosis-related protein markers and the products of free radical reactions. In summary, these results help us to not only understand the underlying mechanisms regarding the protective effect of 14,15-EET, but also broaden our knowledge about 14,15-EET, which may serve as a potential drug to treat ischemic brain injury.

Cerebral ischemia adversely affects vascular function as well that can cause secondary brain injury and limit treatment that depends on a patent vasculature. In contrast, brain parenchymal arterioles retain considerable tone during I/R that likely contributes to expansion of the infarct into the penumbra. Microvascular dysregulation also occurs during ischemic stroke that causes edema and hemorrhage, exacerbating the primary insult. Ischemic injury of vasculature is progressive with longer duration of I/R (Palomares and Cipolla, 2011). As a component of neurovascular unit, CSMC respond actively to acute ischemia and initiate processes that are crucial for subsequent injury events.

I/R-induced CSMCs injury plays a critical role in determining tissue survival after recanalization in focal cerebral ischemia by disrupting the blood-brain barrier integrity and promoting microcirculatory clogging (Palomares and Cipolla, 2011). CSMCs are also important for vascular and tissue remodeling (del Zoppo and Mabuchi, 2003). Ultimately, the proliferation of smooth muscle cells for the development of collateral flow conductance and the remodeling of collateral vessels follow arteriogenesis (Heil and Schaper, 2004). During the late phase of arteriogenesis, ECs and SMCs proliferate

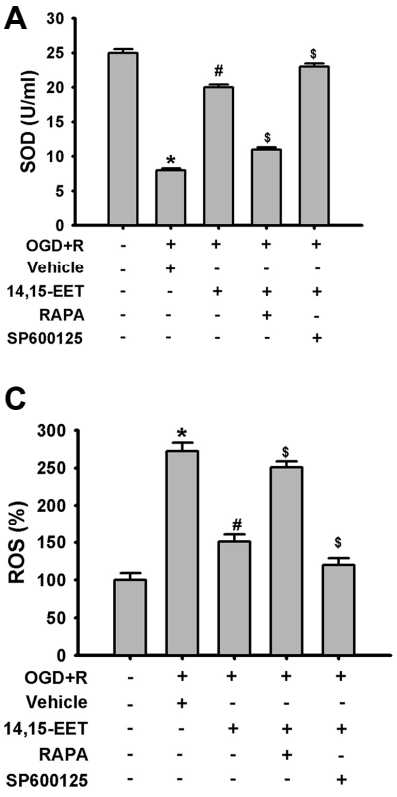

Fig. 5. The MDA content and ROS levels were lowered by 14,15-EET while the SOD activity was increased in OGD-induced CSMC; 14,15-EET' effect was mediated by the JNK inhibitor (SP600125) and mTOR inhibitor (RAPA). The cells were treated with 1 μM 14,15-EET, 10 nM RAPA or 5 μM SP600125 for 30 min before exposing to oxygen glucose deprivation. Reoxygenation was carried out for 24 h following 4-h exposure to hypoxia. (A) Total SOD activity. (B) MDA content. (C) ROS levels. The data were presented as means ± standard deviation; $n = 3$. $*P < 0.05$, compared to controls; $^{\$}P < 0.05$, compared to OGD+14,15-EET; $^{\#}P < 0.05$, compared to OGD+vehicle.

and migrate (Scholz et al., 2000). Therefore. the successful development of targeted therapies for stroke likely requires vascular protection as well as neuroprotection (Palomares and Cipolla, 2011).

There is little evidence concerning the effect of EETs on vascular smooth cells in the brain. Here, CSMCs were targeted in our study. In our previous study, we only showed that 14,15-EET can protect the CSMCs from OGD-induced apoptosis (Qu et al., 2015). Based on previous experiments, the protective effects of three isoforms of EETs (8,9-, 11,12- and 14,15-EET) were compared. The data from the current study demonstrated that among the three isoforms of EETs, 14,15-EET had the most significant effects on CSMCs. However, the cytoprotective functions of 14,15-EET in CSMCs had not been clarified in details until now.

Studies have proposed that oxidative stress results in brain cell damage after I/R injury (von Leden et al., 2016). Furthermore, reoxygenation to ischemia brain damage causes a more severe cerebral injury as compared to that caused by ischemia only (Granger and Kvietys, 2015). I/R injury induces the overproduction of ROS and MDA, which cannot be efficiently eliminated by endogenous antioxidant systems. The accumulation of ROS may cause oxidative damages, leading to brain dysfunction and cell apoptosis (Charriaut-Marlangue, 2004; Takuma et al., 2004). SOD, a key endogenous enzyme with antioxidant features, is one of the important markers for the scavenging capability of free radicals (Briones and Touyz, 2010; Lakshmi et al., 2009). SOD protects tissues against free radicals as well as other oxygen species. Therefore, in the present study, MDA and ROS levels, as well as SOD activity, were estimated in CSMCs after OGD injury. Our results revealed a significant elevation in MDA and ROS levels and decreased SOD activity in CSMCs after OGD injury. However, pretreating the cells with 14,15-EET could significantly decrease the elevated levels of cerebral MDA and ROS while restoring the SOD activity. Therefore, it is likely that EETs can protect against I/R injury by attenuating oxidative cerebral damage.

Growing evidence has shown that numerous molecular and cellular signaling pathways can manage neuronal homeostasis after ischemic injury. Many signal transduction molecules participate in neuroprotection via regulating apoptotic factors including Bcl-2, Bax and caspase-3 (Charriaut-Marlangue, 2004). Bcl-2 exhibits anti-apoptotic functions and is expressed on the extine of mitochondria. Bax, a pro-apoptotic protein, is located inside the protoplasm and can promote apoptosis (Brooks and Dong, 2007). As a pair of harmonious molecules, bcl-2 and Bax both participate in brain injuries by preventing or accelerating cerebral apoptosis in post-stroke stages (Schmidt-Kastner et al., 2000). Another novel finding of this report was that the protective features of 14,15-EET were closely associated with a reduction of apoptotic events known to develop during cerebral ischemia. It was found that Bax and caspase-3 expressions were decreased by 14,15-EET pretreatment while the Bcl-2 expression was increased, strongly supporting the viewpoint that 14,15-EET could improve OGD-induced injury by, at least partially, interfering with the apop-

tosis pathway.

Recent studies have indicated the JNK/c-Jun pathway is involved in the injury responses of stroke (Iyer and Brown, 2009; von Leden et al., 2016). One study has shown that, during focal cerebral ischemia, upregulated phospho-JNK was located in apoptotic neurons that were TUNEL-positive, and the suppression of JNK can be beneficial to the injured brain tissues due to ischemia (Okuno et al., 2004). In addition, several neuroprotectants, such as SP600125 (Chung et al., 2000; Selimi et al., 2000), exert their protective functions through inhibiting the JNK pathway. Ma and colleagues found that EETs treatment promoted the transition of cells from the G0/G1 phase to the S phase and accelerated *in vitro* tube formation; these effects were suppressed after using siRNA targeting JNK1/2 or SP600125 to block the JNK pathway (Ma et al., 2012). In this study, we found that 14,15-EET reduced c-jun and JNK activation in CSMC undergoing OGD. Notably, to investigate whether the JNK/c-jun pathway participated in mediating the protective functions of 14,15-EET in OGD-induced injury of CSMC, SP600125 was used to block the JNK/c-jun pathway. As a result, the SOD activity was increased while the MDA content and ROS levels were decreased when compared with the values in the 14,15-EET+OGD+R group, and the protective functions of 14,15-EET in terms of DNA fragmentation were even further potentiated. In addition, in the presence of the JNK inhibitor, Bcl-2 expression was upregulated while the Bax level was suppressed relative to that in the 14,15-EET+OGD+R group. Furthermore, caspase-3 activity was down-regulated as compared to the value in the 14,15-EET+OGD+R group. The above data showed that the protective effects of 14,15-EET were attenuated by the JNK inhibitor (Figs. 4 and 5). Our study indicated that inhibition of the JNK/c-Jun pathway was the key of EETs' protective functions in ischemic brain injury.

mTOR, a crucial regulator of protein synthesis, participates in other physiological functions, including cell proliferation and growth. mTOR may be involved during ischemic injury because some studies have suggested that the inhibitor of mTOR pathway (RAPA) results in protective effects during I/R (Tatton, 2000). Koh and colleagues found that ischemic brain injury induces a down-regulation of mTOR and p70S6 kinase phosphorylation (Sawada et al., 2000). Whether the activation or inhibition of mTOR mediates important and intermediate interactions following ischemic diseases remains controversial. To investigate whether EETs exert their protective effects through the mTOR pathway during cerebral ischemia, mTOR expression in OGD-induced CSMC was measured. Here we demonstrated that mTOR was significantly down-regulated in OGD-induced CSMCs, consistent with the previous studies. After the application of 14,15-EET, the mTOR protein level was higher. We also found that 14,15-EET prevented the injury-induced decrease in terms of mTOR phosphorylation. Moreover, upon blocking the mTOR pathway, the activity of SOD was decreased while the MDA content and ROS levels were increased as compared to the values in the 14,15-EET+OGD+R group. In addition, the protective functions of 14,15-EET regarding DNA fragmentation were suppressed. In the presence of the mTOR inhibitor, the expression of Bcl-2 was decreased while the Bax level was increased relative to the values in the 14,15-EET+OGD+R group. Additionally, caspase-3 activity was upregulated as compared to the values in the 14,15-EET+OGD+R group. These data suggest that 14,15-EET can inhibit cell apoptosis induced by ischemic brain injury. In addition, the neuroprotective effects of 14,15-EET are achieved by avoiding injury-induced suppression of mTOR phosphorylation.

Whether there is any connection or cross-talk between the JNK and mTOR signal pathway remains to be further explored. The limited data from different studies have addresses the possible link of the two signaling pathways. Fujishita and colleagues found that, in cells of intestinal tumor, activation of mTORC1 requires the involvement of JNK, and both JNK and MTOR signaling pathways possibly function along with factors affecting disease procession (Fujishita et al., 2011). In another study on the mechanism of cadmium-induced neurodegeneration, Chen and colleagues have found inhibitors of JNK and mTOR may have a potential role in preventing cadmium-induced neurodegeneration. Cadmium-induced neurodegeneration is related to the activation of both JNK and mTOR signaling pathways (Chen et al., 2008). Similarly, the results obtained by Benoit and colleagues have shown that, in LS174T colon cancer cells, the efficacy of rapamycin in tumor treatment is reduced by rapamycin-induced JNK phosphorylation and activation (Benoit et al., 2011). In order to perform a general survey regarding the crosstalk between mTOR and JNK signaling pathways in various physiological and pathological processes, we carried out the KEGG pathway analysis (data not shown). We found that in the INSULIN RESISTANCE process, the insulin receptor substrate 1 may act as the intermediate link between the mTOR and JNK signaling pathways. In addition, in the entire ANIMAL-AUTOPHAGY system, both hypoxia and nutrient starvation (amino acids and glucose) could trigger the activation of mTOR and JNK signaling pathways. The data above indicated that there might be some connection or cross-talk between the JNK and mTOR signaling pathways, but research investigating possible relationships is still in its early stage. Our preliminary results revealed that both the JNK and mTOR signaling pathways mediated the expressions of caspase-3, Bax and Bcl-2 while exerted influence on oxidative stress at the same time. The findings indicated that there may be a cross-talk between the JNK and mTOR signaling pathways in CSMC.

As a matter of fact, our research was conducted *in vitro*, thus the outcomes may not be applicable in humans at the present stage. Nevertheless, these findings have suggested new paths for further studies in order to determine whether the observed anti-apoptotic features of 14,15-EET in CSMC are also relevant under clinical settings.

In summary, here we have demonstrated that 14,15-EET plays an important role in promoting the proliferation and inhibiting the apoptosis of CSMCs *in vitro* under OGD conditions. The underlying mechanism involves regulation of the JNK/c-Jun and mTOR signaling pathways. Together these findings suggest that 14,15-EET may become a promising drug for the management of CSMCs and associated patho-

logical processes.

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