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ORIGINAL PAPER

Acetyl-11-keto-β-boswellic acid (AKBA) Attenuates Oxidative Stress, Inflammation, Complement Activation and Cell Death in Brain Endothelial Cells Following OGD/Reperfusion

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Abstract

Brain endothelial cells play an important role in maintaining blood flow homeostasis in the brain. Cerebral ischemia is a major cause of endothelial dysfunction which can disrupt the blood–brain barrier (BBB). Oxygen–glucose deprivation (OGD)/reperfusion promote cell death and BBB breakdown in brain endothelial cells. Acetyl-11-keto-β-boswellic acid (AKBA), a biologically active phytoconstituent of the medicinal plant *Boswellia serrata*, has been shown to be protective against various inflammatory diseases as well as ischemic brain injury. The molecular mechanisms underlying these beneficial characteristics of AKBA are poorly understood. We subjected bEND.3 cells to OGD/reperfusion to investigate the protective role of AKBA in this model. We found that AKBA treatment attenuated endothelial cell death and oxidative stress assessed by means of TUNEL assay, cleaved-caspase-3, and dihydroethidium (DHE) staining. Furthermore, OGD downregulated tight junction proteins ZO-1 and Occludin levels, and increased the expressions of inflammatory cytokines TNF-α, ICAM-1, and complement C3a receptor (C3aR). We also noticed the increased phosphorylation of ERK 1/2 in bEND.3 cells in OGD group. AKBA treatment significantly attenuated expression levels of these inflammatory proteins and prevented the degradation of ZO-1 and Occludin following OGD. In conclusion, AKBA treatment provides protection against endothelial cell dysfunction following OGD by attenuating oxidative stress and inflammation.

Keywords bEND.3 · AKBA · OGD · BBB · Inflammation · Complement C3a receptor

Introduction

The occlusion of a cerebral vessel results in an ischemic stroke, one of the leading causes of death and the leading cause of disability worldwide (Sidney et al. 2018; Writing Group et al. 2016). During an ischemic insult, vascular endothelial cells serve as a primary target for injury, resulting in vascular cell death and dysfunction (Liao et al. 2016; Tornabene and Brodin 2016). Endothelial dysfunction has been implicated in various diseases including diabetes, hypertension and atherosclerosis (Frey et al. 2009). Endothelial cell injury degrades blood brain barrier (BBB) function which maintains the homeostasis of the central nervous system (CNS). BBB dysfunction has also been implicated in the pathophysiology of neurovascular disorders including cerebral ischemia (Huang et al. 2013; Salvador et al. 2015). Tight junction proteins (TJPs) such as occludin and zonula occludens-1 (ZO-1) are the crucial proteins responsible for maintaining barrier integrity (Pan et al. 2017; Strazielle and

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Ghersi-Egea 2013), and loss of these proteins occurs in settings of BBB permeability. Cerebral endothelial dysfunction represents an attractive therapeutic target to minimize brain injury in stroke.

Acetyl-11-keto-β-boswellic acid (AKBA), a bioactive pentacyclic triterpene molecule derived from the gum resin of the medicinal plant *Boswellia serrata*, is used in holistic medicine to treat several diseases such as respiratory, rheumatic, and liver disease (Takada et al. 2006). The useful biological actions of Boswellia extract are attributed to Boswellic acids, predominantly AKBA, which exhibits potent antioxidant and anti-inflammatory properties (Beghelli et al. 2017; Cuaz-Perolin et al. 2008). Furthermore, AKBA attenuates neuronal cell death following OGD and is neuroprotective via Nrf2/HO-1 pathway activation (Ding et al. 2014; Sadeghnia et al. 2017). A recent study suggested that nanoformulation of AKBA could enhance neuroprotection in stroke by improving antioxidant defense and inhibiting inflammation (Ding et al. 2016). Interestingly, AKBA also protects the endothelium and positively regulates the vascular response to inflammation (Cuaz-Perolin et al. 2008; Roy et al. 2006; Wang et al. 2015). However, the mechanisms underlying AKBA-mediated endothelial protection remain unknown.

In the present study, we utilized an in vitro model of cerebral endothelial ischemic injury to test the hypothesis that the potent anti-inflammatory effects of AKBA mediate protection against OGD-induced cerebral endothelial cell death and dysfunction.

Materials and Methods

Chemicals and Reagents

Immortalized bEnd.3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). AKBA (5 mg; source, *B. serrata*; HPLC grade) was procured form Enzo Life Sciences, Inc. NY, USA (ALX-350-310; Lot number: 03161662). Dulbecco's modified Eagle medium (DMEM/High Glucose, GE, Utah, USA), Pen strep (Gibco, USA), FBS (VWR, USA) and all reagents used were AR and molecular grade. TUNEL assay kit was procured from AAT Bioquest (USA). Antibodies (monoclonal antimouse Caspase3; monoclonal antimouse C3aR; monoclonal antimouse ICAM-1; monoclonal antimouse ZO-1 and antimouse Occludin were purchased from Santa Cruz Biotechnology, CA, USA. Lectin was received from Vector Lab (USA). Dihydroethidium (DHE) and Protease inhibitor cocktail was purchased from Sigma (MO, USA). RIPA extraction buffer for Western blotting experiment was procured from Fisher Scientific (USA). All secondary fluorescent antibodies were purchased from Invitrogen (USA).

Oxygen–Glucose Deprivation (OGD/Reperfusion, Cell Culture, and Treatment

OGD/reperfusion is used as an in vitro model to mimics in vivo ischemia/reperfusion injury. The brain endothelial cell culture was performed as per the standard procedure (Zhao et al. 2017). In brief, the immortalized mouse brain endothelial cells (bEnd.3) were cultivated in 25 cm^2 tissue culture flask containing DMEM with high glucose (4.5 mg/ml), supplemented with 10% FBS, and antibiotic. When the cells reached subconfluency, the cells were harvested with trypsin and seeded in 12 well plates (10,000 cells/well) as well as cover slip for immunofluorescence staining. The cells were incubated in a CO_2 incubator with 5% CO_2 at 37 °C. bEND.3 cells were deprived of glucose and oxygen [we changed the culture medium to oxygen$^-$, glucose-free balanced salt solution (EBSS-24010-043, Gibco, USA)] by transferring into an anaerobic chamber (0% O_2) for 3 h. Following OGD, cells were reperfused to a normoxic incubator under 5% CO_2/95% air for 24 h in complete medium. Normoxic bEnd.3 cells served as controls. AKBA (5 & 20 μM in 0.1% DMSO) was added 1 h prior to OGD. In the control group, volume equivalent to 0.1% DMSO was added as vehicle.

TUNEL Assay

To assess the protective effects of AKBA against apoptotic cell death, a commercially available TUNEL assay kit was purchased from Roche (Germany). bEnd.3 cells were cultured on glass coverslips and grown to confluency. Then, the cultured coverslips were subjected to either OGD/reperfusion or normoxic conditions in glucose-free media (Gibco, USA DMEM—glucose—L-glutamine—phenol red), with or without 5 or 20 μM AKBA (0.1% DMSO 1x PBS). After 3 h all groups were returned to complete media with high glucose (4.5 g/L) for 1 h. Next, the cells were washed with PBS, fixed for 5 min in 4% PFA at room temperature, and washed again three times with PBS. Subsequently the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. The coverslips were incubated with the TUNEL Assay kit reagents according to the manufacturer's instructions for 1 h at 37 °C protected from light. Following the labeling of apoptotic DNA fragments, the coverslips were mounted to slides with Vectashield Hard Set Mounting Medium containing DAPI (Vector Laboratories, USA) in order to counterstain cell nuclei. Finally, the slides were analyzed using fluorescent microscopy (Keyence BZ-X, Japan) at × 20 magnification. Images of 10 discrete fields of cells from each coverslips of each group (10 images form each; $n = 4$) were processed and manually scored for TUNEL+ DAPI+ merge signals versus only DAPI+ signals by a technician blinded to sample identity. The average ratio of TUNEL+ DAPI+ cells to only DAPI+ cells was used to calculate the percentage of apoptotic cells.

Dihydroethidium (DHE) Staining for the Detection of ROS

The detection of ROS formation in the cells was performed as previously described (Ahmad et al. 2013). Dihydroethidium (DHE) is used as florescence probing for the ROS detection (specific for superoxide and hydrogen peroxide) and ROS generation was determined as total DHE florescence. In brief, frozen coverslip with bEND.3 cells were used for DHE staining with and without OGD along with control. Cells were incubated with (DHE; 10 μMol/L in PBS) for 30 min at 37 °C in a humidified chamber protected from light. Following incubation, cells were washed with 1 × PBS. DHE emits red fluorescence because DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus. For the detection of ethidium, samples were examined under the fluorescence microscope (Keyence, USA; Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence intensity was quantified in a blind manner using Image J software (NIH). In brief, we took four coverslips from each group (10 images form each), and color intensity was measured by Image J. Through analyze table, we measured the histogram value, and the mean was taken for further analysis to plot the graph using PRISM software.

Immunocytochemistry

bEnd.3 cells were cultured on glass coverslips as described above. Following 1 h of reperfusion, the coverslips were fixed for 20 min in 4% PFA at room temperature before washing with PBS three times and blocking in 10% Normal Goat Serum in PBS-T with 0.5% Triton X-100 for 1 h. Cells were then labeled with primary antibody diluted in 1% BSA 1 × PBS-T with 0.1% Triton X-100 at 1:50 overnight at 4 °C in a humidified, staining chamber. Primary antibody targets Caspase-3, C3aR, pERK1/2, and ICAM-1 were used to assess the levels of apoptosis and inflammatory markers, while tight junction markers ZO-1 and occludin were used to assess monolayer integrity. Caspase-3 and pERK1/2 were rabbit monoclonal antibodies purchased from Cell Signaling Technologies, while C3aR, ICAM-1, occludin and ZO-1 mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology. After primary antibody labeling, fluorescein-conjugated or Texas Red secondary antibody was applied using the same diluent as above for 45 min at room temperature. For the coverslips stained for ZO-1 expression, a 1:1 mixture of mounting medium containing DAPI and Phalloidin (Vector Laboratories, USA) was used to visualize nuclei and F-actin for cytoskeletal integrity. All other coverslips were mounted with only DAPI. The slides were then analyzed using fluorescent microscopy (Keyence BZ-X, Japan) at × 40 and × 20 magnification. Images of four discrete fields of cells from each coverslips of each group ($n = 4$) were analyzed and color intensity was quantified by Image J software (NIH). In brief, we took four coverslips form each group (ten images form each) and color intensity was measured by Image J in blind manner. Through analyze table, we measured the histogram value, and the mean was taken for further analysis to plot the graph by using PRISM software.

Western Blot

bEnd.3 cells were cultured to confluency in poly-D-lysine coated 6 well plates and groups were treated as described above. Following reperfusion, the cells were lysed for 45 min on-ice in RIPA Buffer (Pierce, ThermoFisher, USA) with 1% HALT Phosphatase Inhibitor cocktail (Pierce, ThermoFisher, USA), and 1% Proteinase inhibitor cocktail (Sigma-Aldrich, USA). Samples were then centrifuged at 12,000×*g* for 30 min at 4 °C to remove insoluble material. Protein concentration was quantified using the Bradford Coomassie reagent. Equal masses of protein were loaded onto 4–20% SDS-Page Mini Protean TGX gels (Bio-Rad Laboratories, USA) and PAGE was run at 80 V for 70 min. The gel was then transferred onto PVDF membrane using the Bio-Rad Trans-Blot Turbo system. The membrane was then washed three times in TBST, blocked in 5% skim milk in 1 × TBS-T for 1 h, and then probed with primary antibodies (Mouse monoclonal anti-ZO-1 and Occludin, SCBT and rabbit monoclonal pERK1/2, CST) at a 1:1000 overnight at 4 °C with gentle agitation. The next day, the membranes were washed three times and then probed with HRP-conjugated secondary antibodies (goat antirabbit and antimouse, Sigma, USA) at a dilution of 1:5000. The probes were then visualized using Super Signal West Pico PLUS Chemiluminescent Substrate from ThermoFisher. Membranes were incubated with the Luminol-peroxide mixture for 5 min before imaging the blots on a LICOR Odyssey Fc system. The same membranes were then stripped and re-probed for β-actin to ensure uniform protein concentrations. Quantitation of immunoreactivity was performed using densitometry analysis in standard fashion (Image J software/NIH).

Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α expression in the supernatants of culture media from OGD/reperfused bEND.3 cells was determined by ELISA kits (R&D, Minneapolis, MN) as per the manufacturer's instructions. Standards and unknown samples were added and bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by substrate solution, which yielded a color product. The intensity of color was measured by Tecan infinite 200 plate reader (Switzerland) at wavelength 450 nm. The values were calculated from the standard curve and corrected for protein concentration.

Statistical Analysis

Quantitative data are presented as mean ± SEM. Differences among experimental groups were determined by analysis of variance (one-way ANOVA) followed by Newman-Keuls multiple comparison tests. Significance was defined as $p < 0.05$. Data were analyzed using Graph Pad PRISM software.

Results

AKBA Treatment Attenuates bEND.3 Cell Death Following OGD/Reperfusion

We used cleaved-caspase-3 and TUNEL staining to evaluate the effect of AKBA on OGD-induced cell death. We found that OGD triggers cell death by increasing cleaved-caspase-3 expression levels and TUNEL apoptotic cells respectively (Fig. 1a–d). Notably, AKBA treatment (using two different doses; 5 and 20 µM) attenuated cell death assessed by Caspase-3 expression and TUNEL staining following OGD () ($p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$).

AKBA Suppresses Reactive Oxygen Species (ROS) Formation Induced by OGD/Reperfusion in bEND.3 Cells

Reactive oxygen species (ROS) are produced endogenously in response to inflammation and shear stress in endothelial cells. Excess generation of ROS, such as superoxide (O_2^-), contributes to brain endothelial cell death (Frey et al. 2009). OGD/reperfusion significantly increased the ROS determined by dihydroethidium (DHE) staining in bEND.3 cells and AKBA treatment (20 µM) attenuated formation of ROS (Fig. 2a–d; $p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$) in this model.

AKBA Administration Attenuates the Diminished Tight Junction Proteins ZO-1 and Occludin Following OGD

It is well established that TJPs are responsible for maintaining BBB integrity (Ma et al. 2013). Zonula occludens-1 (ZO-1) and Occludin is a predominant protein in tight junction complexes. Immunohistochemistry and Western blot showed that OGD causes downregulation of ZO-1 and Occludin expression respectively in bEND.3 cells which represents BBB disruption during vascular injury. We found that AKBA treatment significantly increased ZO-1 and Occludin expression following OGD (Fig. 3a–f; $p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$).

AKBA Reduces Expression of TNF-α and ICAM-1 in bEND.3 Cells

We next evaluated the expression level of inflammatory markers (TNF-α/ICAM-1) in endothelial cells. These proteins are expressed by ischemic endothelial cells and contribute to vascular cell death and dysfunction (Chen et al. 2017; Guo et al. 2010; Yin et al. 2002). We evaluated TNF-α expression using ELISA and ICAM-1 by immunohistochemistry and found that OGD significantly increased their expression levels, but AKBA treatment attenuated the expression of these inflammatory markers. However, we did not see any significant difference in TNF-α expression comparing control versus vehicle and drug control. This illustrates the potent anti-inflammatory effect of AKBA on endothelial cells (Fig. 4a, b; $p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$).

AKBA Reduces Complement C3a Receptor (C3aR) Activation in bEND.3 Cells Following OGD

Complement activation is recognized as a key player in several neurodegenerative and inflammatory disorders. Specifically, the C3a anaphylatoxin is plays an important role in endothelial activation in brain (Wu et al. 2016). Recently, we and others have shown increased C3aR expression in ischemic brain tissue and endothelial cells (Arumugam et al. 2009; Van Beek et al. 2000; Zhao et al. 2017). We now demonstrate that AKBA treatment suppresses OGD-induced expression of endothelial C3aR. This suggests that AKBA may modulate endothelial complement receptor expression and suppress complement-mediated inflammation following experimental ischemia (Fig. 5; $p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$).

AKBA Attenuates pERK ½ Expression Induced by OGD in bEND3 Cells

OGD activates of downstream extracellular signal-regulated kinase (ERK) in endothelial cells. ERK signaling has been implicated in endothelial cell death following ischemic injury (Narasimhan et al. 2009). Our histochemical and

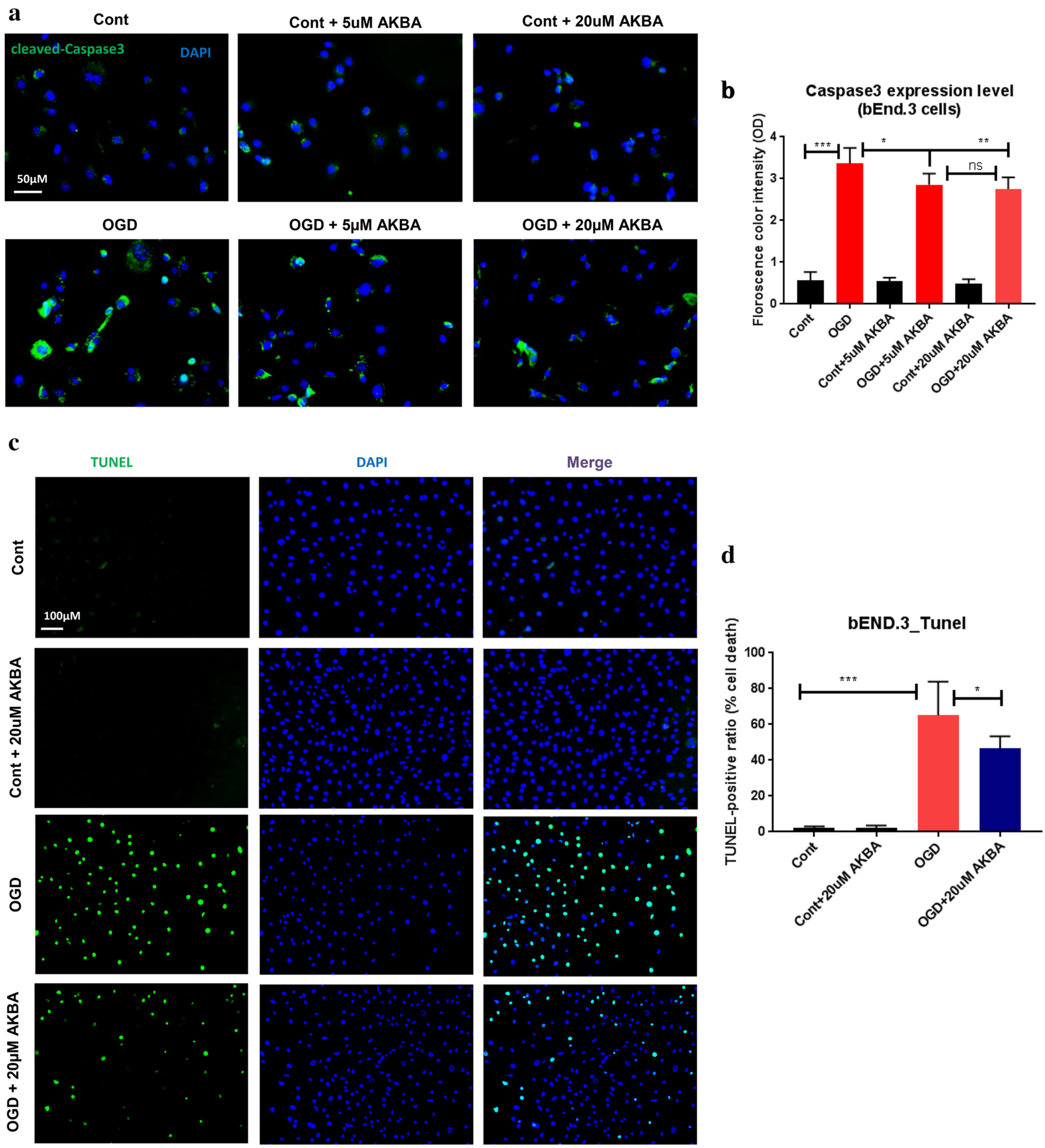

Fig. 1 AKBA treatment causes reduction of bEND.3 cell death following OGD/reperfusion. **a**, **b** Cleaved-caspase-3 and **c**, **d** TUNEL immunostaining was performed to show endothelial apoptosis. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining. Fluorescence color intensity was assessed using Image J (NIH). Data shown are the mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

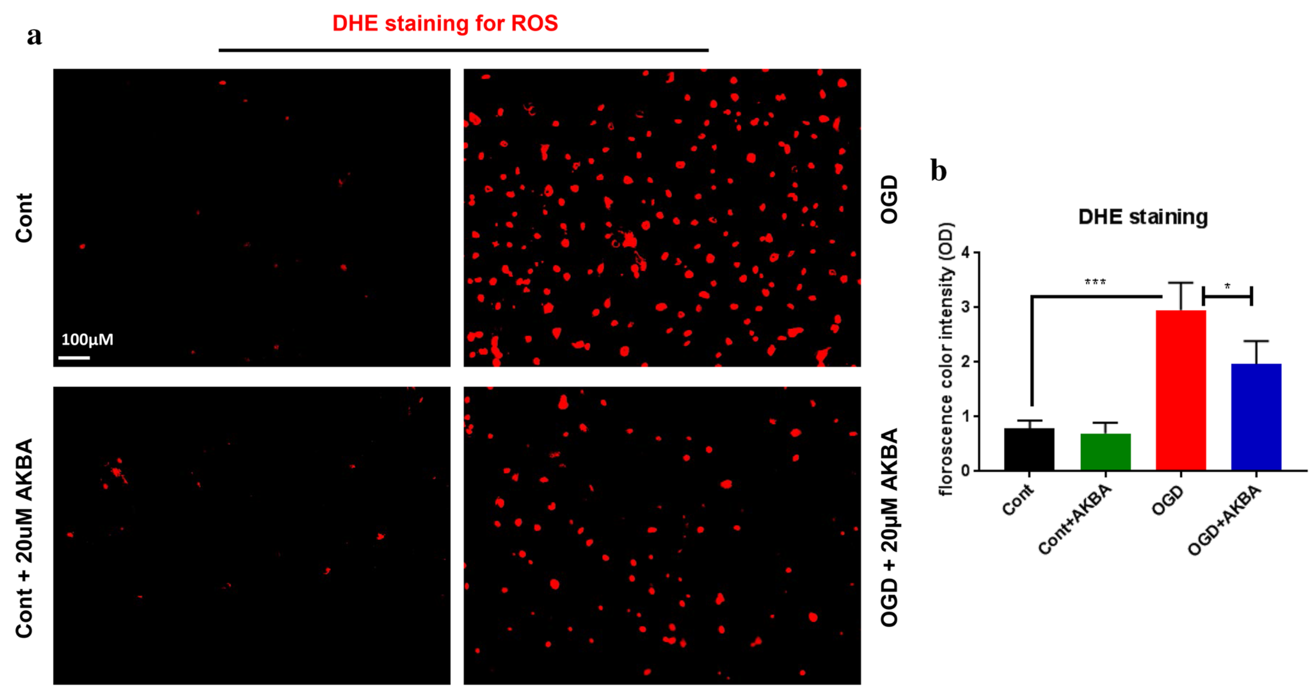

Fig. 2 AKBA reduces ROS formation in bEND.3 cells following OGD. **a** Effect of AKBA on ROS formation was determined by dihydroehidium (DHE) staining using immunocytochemistry. **b** DHE color intensity was measured by Image J (NIH). The results represent the mean ± SEM of fold changes ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Western blot data demonstrate that OGD increased the phosphorylation of ERK 1/2 phosphorylation and AKBA treatment significantly inhibits this process (Fig. 6) ($p < 0.01$, $p < 0.001$, $p < 0.05$) ($n = 4$).

Discussion

Cerebral ischemia causes a disruption of cerebral blood flow that reduces oxygen and glucose supply leading to decreased energy for brain cells. This triggers injury cascades including inflammatory pathways that cause BBB injury and neurovascular degeneration. In the current study, we used OGD/reperfusion as in vitro model of cerebral ischemia in an immortalized mouse brain endothelial cell (bEND.3) line. OGD causes cell death and BBB dysfunction as well as inflammation (Salvador et al. 2015). Over the years, several anti-inflammatory mechanisms have been targeted to improve outcome following brain ischemia, but none of these strategies have successfully translated to the clinic. Nevertheless, there remains considerable interest in the development of anti-inflammatory and antioxidant compounds for the treatment of neurological disease.

The present study probes the mechanisms by which acetyl-11-keto-β-boswellic acid (AKBA) reduces inflammation and oxidative stress, and enhances the integrity of tight junctions in bEND.3 cells following OGD/reperfusion. To test our hypothesis, we first evaluated the effect of AKBA (5 or 20 µM) on OGD-induced cell death and found that AKBA treatment significantly attenuated apoptotic cell death. Likewise, we found that AKBA inhibited reactive oxygen species (ROS) formation in bEND.3 cells following OGD. It is well known that ROS functions as signaling molecules which regulate various biological responses including gene expression, angiogenesis, and apoptosis, as well as the pathophysiology of endothelial dysfunction (Frey et al. 2009). A recent study reported that ROS activates the pro-apoptotic marker caspase-3 following OGD-induced ischemia/reperfusion injuries in microvascular endothelial cells (Alluri et al. 2014). A similar study demonstrated that AKBA is neuroprotective in cortical neuronal culture following OGD by reducing ROS generation and attenuating cell death (Ding et al. 2014).

Ischemia/reperfusion (IR) plays a major role in the pathogenesis of varied diseases including stroke, trauma, vascular cognitive impairment, and myocardial infarction (Alluri et al. 2015; Eltzschig and Eckle 2011). Ischemic injury promotes alteration of BBB tight junctions and vascular permeability resulting in cerebral edema (Alluri et al. 2015; Khatri et al. 2012). Zonula occludens-1 (ZO-1) and Occludin are integral membrane-bound tight junction (TJ) protein which plays specific role in post-ischemic BBB permeability (Ahmad et al. 2019; Jiao et al. 2011; Pan et al. 2017). OGD/reperfusion also induces BBB dysfunction

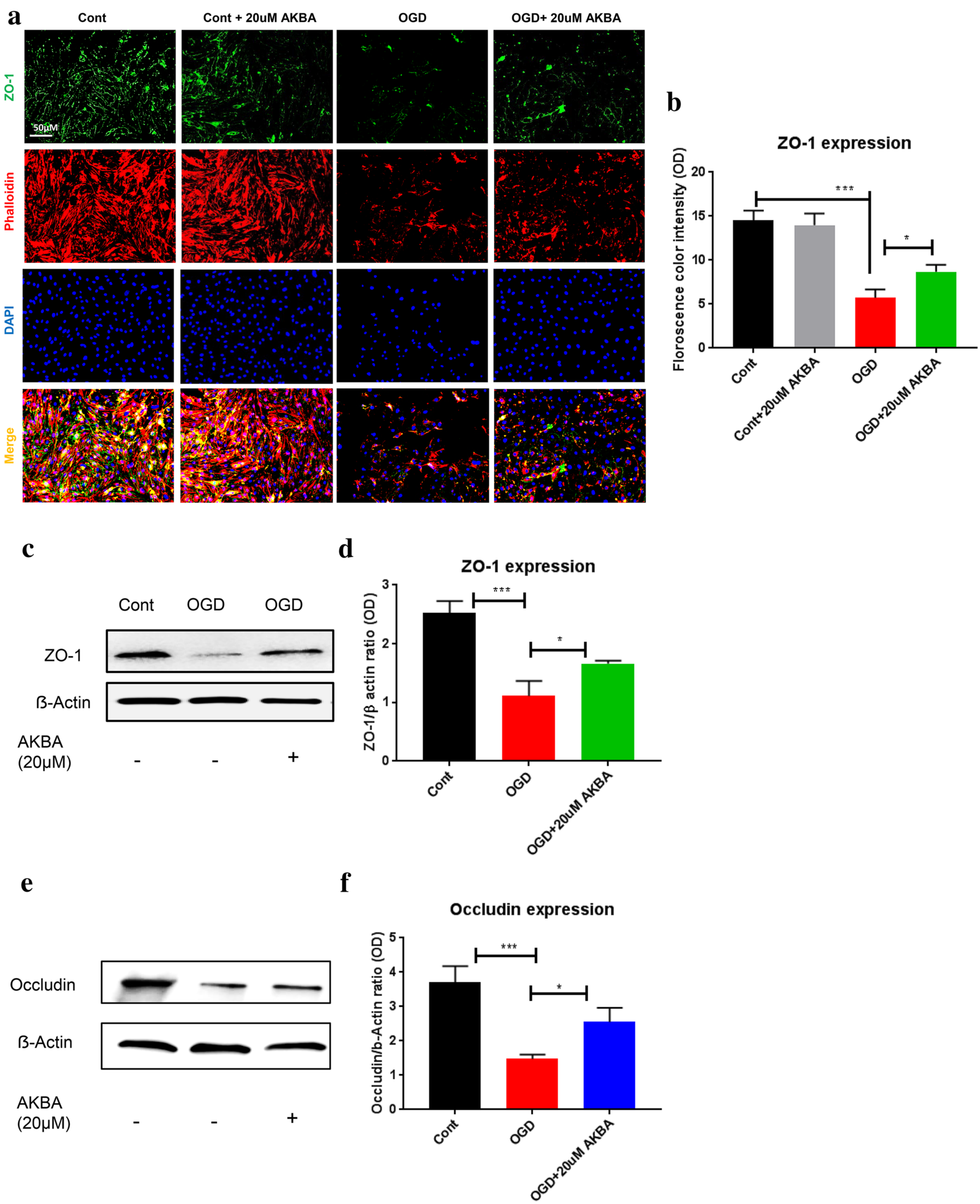

Fig. 3 AKBA attenuates degradation of the tight junction protein ZO-1 and Occludin following OGD. **a–d** ZO-1 expression was assessed using immunocytochemistry and Western blot. **e**, **f** Occludin expression was evaluated by Western blot only, and ZO-1 color intensity and densitometry were performed using Image J software (NIH). Phalloidin labeling (red color) was used to show the cell cytoskeleton. DAPI (4′,6-diamidino-2-phenylindole) is used for nuclear staining. The results represent the mean ± SEM of fold changes ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

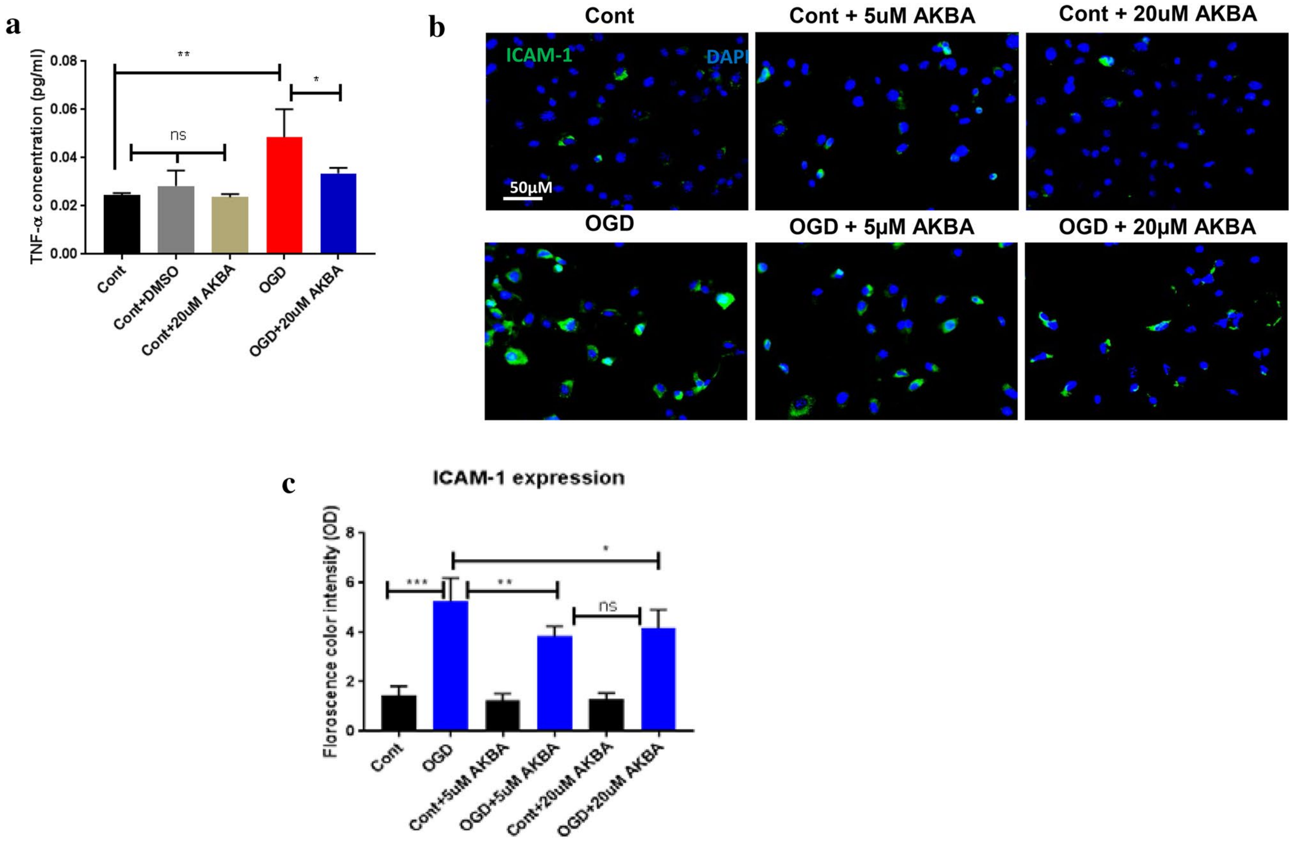

Fig. 4 AKBA reduces expression of TNF-α and ICAM-1 in bEND.3 cells following OGD/reperfusion. **a** TNF-α level in media supernatant was determined by ELISA, and **b** ICAM-1 expression was checked by immunocytochemistry. **c** ICAM-1 fluorescence intensity was monitored by Image J (NIH). DAPI (4′,6-diamidino-2-phenylindole) is used for nuclear staining. Data shown are the mean ± SEM ($n=4$). *$p<0.05$, **$p<0.01$, ***$p<0.001$

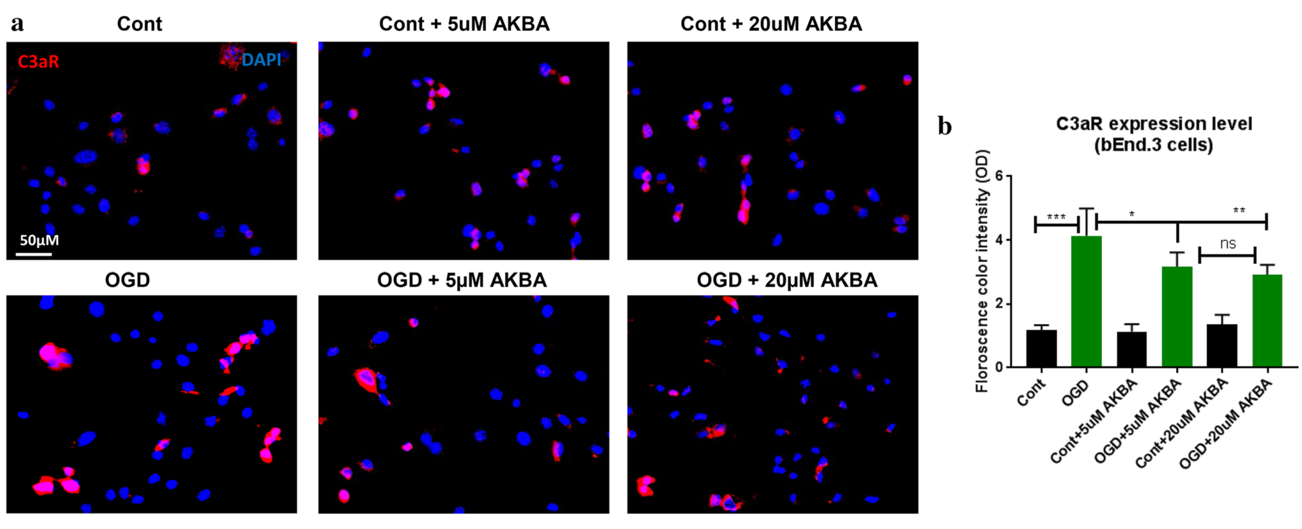

Fig. 5 AKBA treatment abrogates C3aR expression following OGD/reperfusion. **a** Representative fluorescence images depicting C3aR expression in bEND.3 cells. **b** C3aR color intensity was accomplished using Image J (NIH). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining. The results represent the mean ± SEM of fold changes ($n=4$). *$p<0.05$, **$p<0.01$, ***$p<0.001$

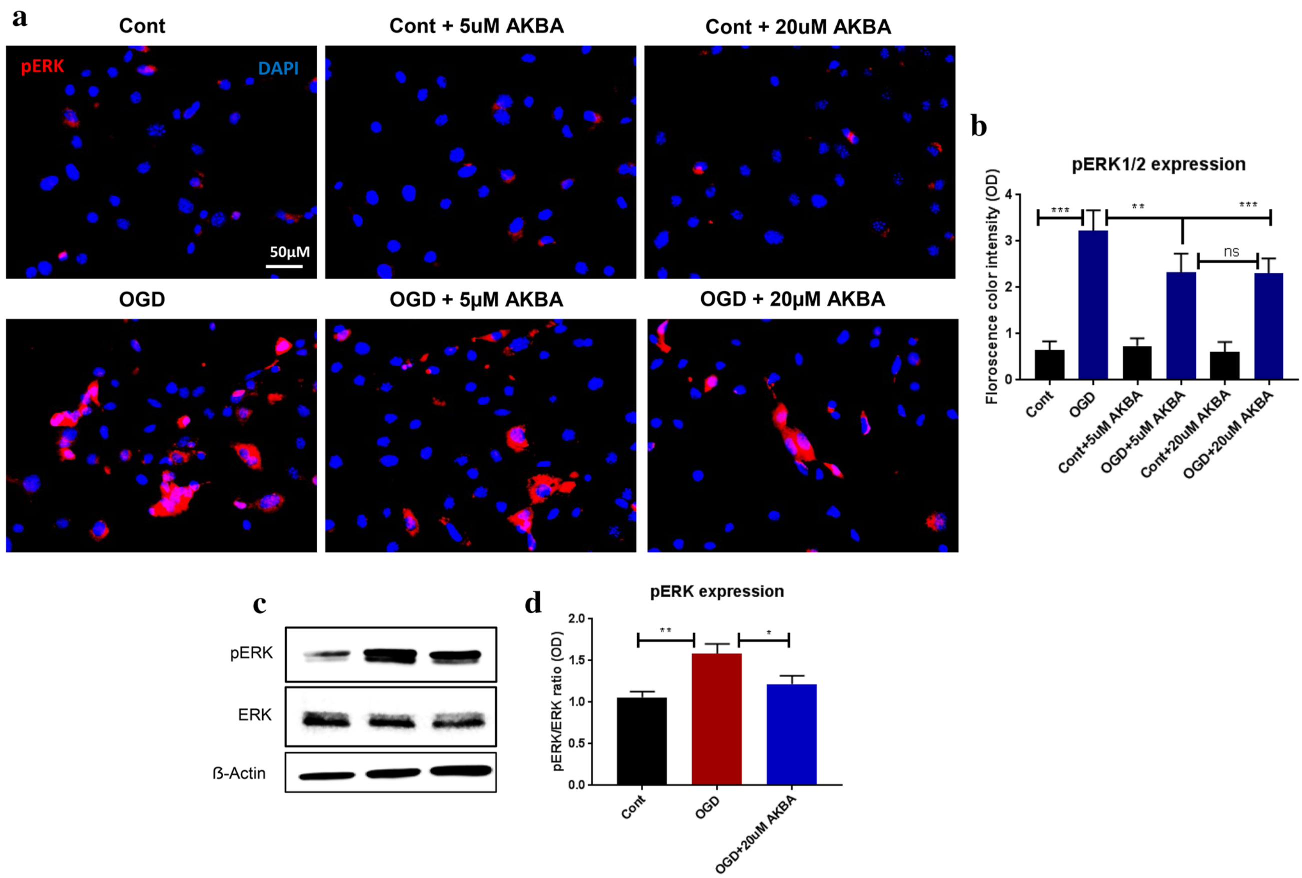

Fig. 6 AKBA treatment inhibits OGD-induced ERK1/2 activation in bEND.3 cells. **a** Phosphorylation of ERK1/2 was observed by immunohistochemistry and **c** Western blot techniques. **b** Fluorescence color intensity and **d** pERK1/2 densitometry analysis was done by Image J (NIH). DAPI (4′,6-diamidino-2-phenylindole) is used for nuclear staining. The results represent the mean ± SEM of fold changes ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

(Alluri et al. 2015), and our data confirm that OGD/reperfusion degrades endothelial ZO-1 and Occludin expression. Recently, we have shown that OGD causes downregulation of Occludin expression in bEnd.3 cells (Ahmad et al. 2019). AKBA attenuated this OGD-induced decrease in ZO-1 expression, suggesting that AKBA may stabilize tight junction following an ischemic insult.

Inflammation underlies the pathophysiology of cerebral ischemia. Pro-inflammatory cytokines, particularly tissue necrosis factor-α (TNF-α) and intracellular adhesion molecule-1 (ICAM-1), drive brain endothelial dysfunction. TNF-α induces ROS formation, apoptosis, and inflammatory injury as well as altered permeability in endothelial cells (Ni et al. 2017; Zhou et al. 2017). The activity of TNF-α is mediated through its surface receptor found on neuron and glial cells as well as endothelial cells (Watters and O'Connor 2011). Similarly, increased ICAM-1 expression has been reported following cerebral ischemia, and is involved in the adhesion and migration of leukocytes along with brain endothelial TJs alteration. Here, our ELISA and immunofluorescence data demonstrate that OGD/reperfusion significantly elevated the expressions of TNF-α and ICAM-1; however, AKBA attenuated the expression of these proteins. This finding confirms the potent anti-inflammatory properties of AKBA, which has previously been shown to inhibit the inflammatory mediator 5-lipoxygenase and NF-kB (Bertocchi et al. 2018; Cuaz-Perolin et al. 2008).

We also investigated the effect of AKBA on endothelial expression of the complement C3aR and found that AKBA significantly reduced C3aR expression following OGD/reperfusion. C3aR has been implicated in pathogenesis of brain ischemic injury, and reports suggest that post-ischemic C3aR inhibition reduces brain hemorrhage and edema (Ducruet et al. 2012). We have previously shown that OGD/reperfusion promotes C3aR activation in bEND.3 cells (Zhao et al. 2017). To the best of our knowledge, the present study is the first to report that AKBA inhibits the expression of C3aR on ischemic endothelium. The present study also showed that AKBA inhibits phosphorylation of extracellular signal-regulated

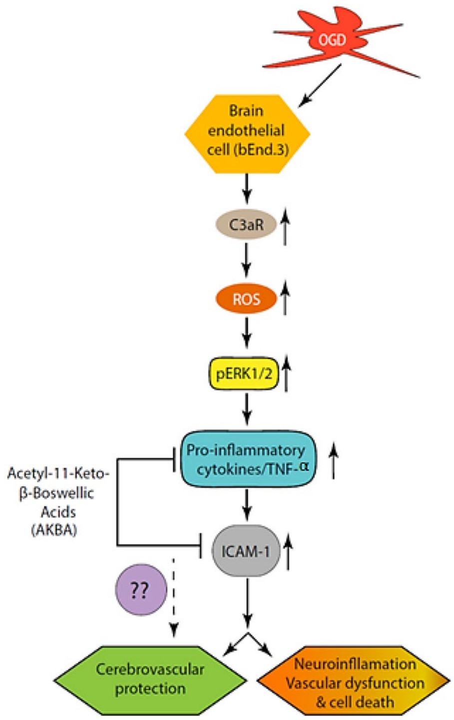

Fig. 7 Schematic diagram depicting OGD-induced inflammation and oxidative stress in bEND.3 cells and its attenuation by AKBA. Putative mechanisms underlying cerebral endothelial cell protection following AKBA administration in the setting of OGD/reperfusion

kinase-1/2 (ERK1/2) following OGD/reperfusion. ERK1/2 activation promotes inflammation and oxidative stress following ischemic insults mediated by pro-inflammatory cytokines. These findings are supported by another study showing that AKBA inhibits ERK1/2 phosphorylation in meningioma cells stimulated by platelet-derived growth factor BB (Park et al. 2002).

AKBA exhibits potent anti-inflammatory and antioxidant effects and has been previously reported to be a neuroprotective agent (Ding et al. 2016). AKBA treatment has also been shown to enhance vascular remodeling in spontaneously hypertensive rats (Shang et al. 2016). In addition, AKBA has been shown to attenuate OGD-induced neuronal cell death and to ameliorate ischemic neuronal injury in an MCAO model (Ding et al. 2014; Sadeghnia et al. 2017). In conclusion, the present study suggests that AKBA attenuates bEND.3 cell death induced by OGD/reperfusion through inhibition of pro-inflammatory signaling molecules, ROS formation, and ERK1/2 activation (Fig. 7). Therefore, AKBA represents a promising natural product to protect ischemic endothelium in stroke.

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Compliance with Ethical Standards

Conflict of interest None of the authors have any conflicts of interest to disclose.

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