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Research Paper

Salvinorin A moderates postischemic brain injury by preserving endothelial mitochondrial function *via* AMPK/Mfn2 activation

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ABSTRACT

Salvinorin A (SA) is a highly selective kappa opioid receptor (KOR) agonist that has significant protective effects on cerebrovascular function after ischemic stroke, but its underlying mechanism is still unclear. This study aimed to investigate whether KOR activation improves the morphology and function of intracellular mitochondria to protect endothelial cells after cerebral ischemia. A transient ischemic brain damage was generated by establishing middle cerebral artery occlusion (MCAO) model in male Sprague-Dawley rats and oxygen glucose deprivation (OGD) model in human brain microvascular endothelial cells (HBMECs). *In vivo* findings revealed that SA significantly reduced the infarct size, brain edema and Evans blue effusion after MCAO. *In vitro* findings revealed that SA improved the cell viability and decreased the apoptotic rates in HBMECs OGD model. SA also protected membrane potential and morphology of mitochondria, reduced the ROS level after OGD. SA function was blocked by KOR inhibitor norbinaltorphimine (NB). SA upregulated the phosphorylation levels of AMPK, and Mfn2 expression. Our findings suggest that SA effectively mitigated focal cerebral ischemic injury by activating KOR which potentially preserved mitochondrial function by up-regulating AMPK/Mfn2 in endothelial cells.

1. Introduction

Stroke is one of the leading causes of disability and death worldwide, and stroke in > 80% patients is caused by ischemia. Cerebral ischemia involves abnormal energy metabolism in the brain cells due to insufficient blood supply to the brain parenchyma around cerebral vessels, resulting in the occlusion of one or more cerebral vessels. Ischemic stroke often leads to irreversible motor and sensory deficits (Enzmann et al., 2018; Liu et al., 2018a; Xing et al., 2017).

Brain is an important organ that consumes high energy, and is rich in mitochondria. As a vital component that maintains blood-brain barrier and tight junctions, cerebrovascular endothelial cells have high demand in energy metabolism too. The influence of mitochondria present in neuronal cells, glial cells and microglial cells on cerebral ischemia has been widely studied, but there were very few studies that reported the role of mitochondria in cerebrovascular endothelial cells (Rutkai et al., 2017). Mitochondria are regarded as the "power station" of cells, and they play a key role in maintaining the energy metabolism of cells and determining the vitality of cells. Mitochondrial dysfunction has been reported to be one of the causes of ischemia/reperfusion (I/R) injury, but much attention has not been received due to its role in cerebrovascular endothelial cells till recently (Rutkai et al., 2017). Mitofusin-2 (Mfn2) is a key factor in mitochondria that regulates the fusion and homeostasis, and plays an essential role in maintaining the morphology and functions of mitochondria (Chun et al., 2018; Huang et al., 2016). For this reason, Mfn2 regulation has been extremely important for mitochondrial functioning. Both *in vivo* and *in vitro* studies showed that after ischemic injury, the increased Mfn2 expression significantly ameliorated the morphology and mitochondrial functions (Chun et al., 2018; Gall et al., 2015; Qin et al., 2018). Therefore, regulation of Mfn2 has become an effective target in therapeutic interventions.

During cerebral ischemia, insufficient local blood flow caused deprivation of oxygen and glucose, leading to the disorder of energy metabolism in cells, disorder of ATP synthesis, excessive production of reactive oxygen species (ROS) and inflammatory reaction, and is

Abbreviations: SA, Salvinorin A; KOR, kappa opioid receptor; MCAO, Middle Cerebral Artery Occlusion Model; AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; OGD, Oxygen glucose deprivation; HBMECs, human brain microvascular endothelial cells; NB, norbinaltorphimine; Mfn2, Mitofusin-2

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reported to have an initial association with ischemia-reperfusion injury (Cao et al., 2017). The adenosine monophosphate-activated protein kinase (AMPK) is an energy-sensitive protein kinase that is widely present in eukaryotes and is a cellular energy sensor (; Miller et al., 2008). When the energy metabolism is disordered, AMPK still plays an important regulatory role. Many studies have shown that AMPK activation after ischemia/reperfusion acts as a key target for initiating the protection (Jiang et al., 2018). Some studies, in particularly, proved that the activation of AMPK can reduce the damage of cerebrovascular endothelial cells caused by cerebral ischemia (Chen et al., 2018). Due to the crucial role of mitochondria in energy metabolism, some studies have explored the influence of AMPK on mitochondria and verified that the activation of AMPK can maintain the biological functions of mitochondria (Hang et al., 2018). The regulation of mitochondrial functions through AMPK energy metabolism pathway might be an important step for preventing cerebrovascular endothelial damage after ischemia/reperfusion.

The κ-opioid receptor (KOR) is widely distributed in the brain tissues of humans and animals, and is highly expressed in cerebral cortex, hippocampus, corpus striatum, *etc.* (Mansour et al., 1995). Many studies have documented that KOR agonists significantly reduced nerve injuries and cerebral edema caused by ischemia/reperfusion and protected the blood-brain barrier function (Chen et al., 2016; Yang et al., 2011). Salvinorin A (SA) is a short-acting highly-selective KOR agonist that easily passes through the blood-brain barrier, causing a small impact on the circulation and wide safety margin. Therefore, it has unique advantages in ischemia/reperfusion injury (Chen et al., 2016). Existing studies have proved that the activation of KOR has a notable protective effect on cerebral vessels after ischemia and hypoxia (Dong et al., 2018; Wang et al., 2012; Xin et al., 2016), but the protective mechanism has not been fully elucidated yet.

Hence, in the present study, a middle cerebral artery occlusion (MCAO) model in rats and a cerebrovascular endothelial cell oxygen-glucose deprivation (OGD) model were generated to discuss the stabilization of mitochondrial functions in the cerebrovascular endothelial cells and the reduction of damage of ischemia/reperfusion in the cerebral vessels through AMPK/Mfn2 signaling pathway after treatment with SA activated KOR.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 220–250 g, were purchased from Laboratory Animal Center, Shanghai Jiaotong University. The animals were maintained in a controlled environment (12:12-h light/dark cycle, RH = 60% ± 5%, 22 ± 2 °C), and had free access to water and food. All the procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Middle Cerebral Artery Occlusion Model

A MCAO model was set up as described previously (Lückl et al., 2018). In brief, the rats were anesthetized by injecting intraperitoneally with 3% pentobarbital sodium (50 mg/kg) (Sigma, St Louis, MO, USA). A surgery was performed to expose their right common carotid artery, internal carotid artery and external carotid artery. A monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd.) with a round needle tip was inserted into the internal carotid artery through the external carotid artery stump and then was gently inserted into the middle cerebral artery. After arterial occlusion for 90 min, the suture was removed to restore blood flow. Similar surgical procedure without a suture was performed for sham control rats. Throughout the surgery, the rectal temperature was maintained at 37 °C by means of a heating pad and a heating lamp.

2.3. Drug treatments

The rats in the sham group received anesthesia and their bilateral common carotid arteries were isolated without any ischemia. In the SA treatment group, SA (Sigma, USA, 20 μg/kg, i.v.) was administered after reperfusion. Administration of SA before 30 min, NB (Sigma, St Louis, MO, USA, 4 mg/kg, i.v) was also given. The drug doses were based on the effective doses used in our previous report (Xin et al., 2016). Thirty minutes prior to MCAO, intracerebroventricular injection of Compound C (CC, Dorsomorphin, Sigma, St Louis, MO, USA, 0.1 μmol, 10 μL) was given to block the function of AMPK as described previously (Li et al., 2017a).

2.4. Neurological deficit assessment

The neurological deficit scores were assessed as reported previously (Kotoda et al., 2018). The scoring criteria were as follows: (0) no movement deficits (normal); (1) the forepaw was weak and the body turned to the ipsilateral side if pulled by the tail (mild); (2) circling towards the affected side, but the posture remained normal at rest (moderate); (3) the affected side cannot bear the weight at rest (severe); and (4) no spontaneous barrel rolling or motor activity.

2.5. Modified neurological severity score (mNSS)

Modified neurological severity score was assessed by an investigator blinded to the experimental groups. On days 1, 3, 5, and 14 after the MCAO operation, the mNSS was used to determine the nerve injury of rats. As described previously (Zhang et al., 2019), mNSS is considered as the evaluation standard for rat movement, reflex, sensation and balance, with a minimum score of 0, and a maximum score of 18. Evaluation standard was as follows: scores 1–6, mild injury; scores 7–12, moderate injury; scores 13–18, severe injury.

2.6. Infarct volume and cerebral edema assessment

After reperfusion for 24 h, the infarct volume was assessed using 2, 3, 5-triphenyltetrazolium chloride (TTC). Euthanasia of rats was performed and their brains were removed immediately. After that, the tissues were cut into 5 coronal sections of 2 mm, stained with 1% triphenyltetrazolium chloride (TTC) solution at 37 °C for 20 min and then were fixed by immersing them in 4% paraformaldehyde. The TTC-stained brain sections were photographed using a digital camera. The digital images were then analyzed using Image-Pro Plus 6.0 software. The lesion volume was calculated by multiplying the lesion area and the slice thickness. The infarct volume was presented as the percentage of hemisphere lesion volume (%HLV) and calculated by the following formula: infarct volume = [(total contralateral hemispheric volume) – (total ipsilateral hemispheric stained volume)]/(total contralateral hemispheric volume) × 100%.

The rats were sacrificed and their brains were removed immediately after 24 h of MCAO. The brain water content was assessed as described in previous reports (Lee et al., 2012). Using a piece of neutral filter paper, the blood stains from the brain were absorbed and cleared. The brain hemispheres were dissected and the wet weight of the tissues was determined using a chemical scale (BS110S, Sartourius, Germany) within 90s. After that, the tissues were dried in a desiccating oven at 110 °C for 48 h to obtain the dry weight. The brain water content was calculated using the following formula: brain water content % = (wet weight- dry weight)/wet weight × 100%.

2.7. Blood-brain barrier function assessment using Evans Blue (EB)

Forty-eight hours after MCAO, 4 ml/kg 2% EB solution (Sigma-Aldrich) was injected into the caudal vein of the rats. After 2 h, the rats were decapitated and their brains were fixed by transcardial perfusion

with normal saline. As reported previously (Ge et al., 2018), the injured hemisphere was quickly dissected, weighed, and incubated in *N*, *N*-Dimethylformamide at 37 °C for 48 h and then centrifuged the tissues at 1000 ×*g* for 15 min. The absorbance of the supernatant was then measured by using a spectrophotometer at a wavelength of 632 nm. EB extravasation was quantified based on the standard curve.

2.8. Immunofluorescenceand imaging

Twenty-four hours after forebrain ischemia, the rats were anesthetized with 3% pentobarbital and intracardially perfused first with 250 mL saline and then with 250 mL 4% paraformaldehyde. The brains were removed, postfixed with 4% PFA for 24 h, transferred to 30% sucrose solution in PBS, embedded in OCT media and then were frozen. Coronal sections were cut (30 μm) using a cryostat and immunofluorescence was conducted in floating sections. Sections were preserved at −200 °C in a PBS solution containing 30% polyethylene glycol and 30% sucrose. Sections were washed thrice with Tris-buffered saline (TBS), blocked with TBS-blocking solution (1% bovine serum albumin, 0.2% skim milk, and 0.3% Triton X-100 in TBS) for 1 h and then incubated with specified primary antibodies: rabbit anti-Mfn2 1:400(Cell Signaling Technology, CST), rat anti-CD31 1:200 (Biosciences) in TBS-blocking solution overnight on a shaker at 4 °C, followed by the addition of appropriate secondary antibodies for 50 min at room temperature. The brain slices were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min and then were mounted onto the slides. Images were then acquired under a fluorescent microscope (× 400 magnifications; AX80, Olympus).

2.9. Cell culture and recognition of human brain microvascular endothelial cells (HBMECs)

The HBMECs were provided by ScienCell Research Laboratories (Carlsbad, California, USA). The HBMECs were seeded on a 100 mm plate (106 cells per well) in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin solution and then were incubated in 5% CO_2 at 37 °C until the cells reached > 90% confluence. Later, the cells were digested using 1 ml trypsin for 2–3 min and centrifuged at 1000 rpm for 5 min. After that, the fresh medium was replenished and passaged into a secondary tissue culture dish. After digestion, the cells were centrifuged at 1000 rpm for 5 min. The supernatant was then removed, and the cells formed a pellet at the bottom of the centrifuge tube. A frozen solution consisting of 10% DMSO, 40% FBS and 50% DMEM was added to the freezer storage bin for cryopreservation.

2.10. Oxygen glucose deprivation (OGD)/reoxygenation cell injury model

The HBMECs were seeded in a new 100 mm culture dish and incubated in an atmosphere of 5% CO_2 and 95% N_2 using an incubator. Before OGD, the culture dish was rejected and the cells were rinsed with phosphate buffer saline (PBS) solution. The HBMECs were then put in an anaerobic chamber (tension = 0.1%). The PBS was rejected and the deoxygenated glucose-free balanced salt solution (BSS) was used instead. The cells were exposed to OGD for 6 h. After OGD, the BSS solution was washed and then the DMEM culture medium was used instead. After that, the cells were moved into a CO_2 incubator for 24 h. After reperfusion, the cells and supernatants were acquired and stored at −80 °C. During reoxygenation, the cells were treated using a concentration of SA (5uM).

2.11. AMPKα siRNA cell transfection

To investigate the role of AMPK, HBMECs were transfected using 100 nM siRNAs that specifically targeted AMPK (si-AMPK) for 24 h before OGD. Transfection was performed using Lipofectamine 2000 reagent according to the manufacturer's instructions. The efficiency of siRNA transfection was assessed by analyzing mRNA expression of AMPK (RT-PCR). Transfection was divided into 4 groups: A. siRNA NC; B. AMPKα siRNA 1; C. AMPKα siRNA 2; D. AMPKα siRNA 3;

Gene	Sequence	
	Sense (5′–3′)	Antisense (5′–3′)
Human AMPKα		
AMPKα siRNA 1	GCGUGUACGAAGGAAGAAUTT	AUUCUUCCUUCGUACACGCTT
AMPKα siRNA 2	GAGGAGAGCUAUUUGAUUATT	UAAUCAAAUAGCUCUCCUCTT
AMPKα siRNA 3	CGGGAUCAGUUAGCAACUATT	UAGUUGCUAACUGAUCCCGTT

2.12. Real-time PCR

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. After extraction, by using the First Strand cDNA Synthesis Kit (Thermo Scientific Inc., Rockford, USA), 500 ng of total RNA was used as a template for synthesizing cDNA. The primers used for AMPK were as follows: forward, 5'-TTGAAACCTGAAAATGT CCTGCT-3′; reverse: 5'-GGTGAGCCACAACTTGTTCTT-3′, while the primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: forward, 5'-AATCCCATCACCATCTTCCAG-3′; reverse: 5'-ATCAGCAGAGGGGGCAGAGA-3′.

2.13. Cell viability assay

The HBMECs were cultured in a 96-well plate and then exposed to OGD conditions. Cell proliferation was measured by using a Cell Counting Kit-8 (Dojindo Laboratories, Japan) according to the manufacturer's instructions. After 2 h of incubation, the absorbance was measured with a microplate reader (Bio-Rad Laboratories, Hercule, USA) at a wavelength of 450 nm.

2.14. Analysis of HBMECs apoptosis by Flow-cytometry

The apoptosis rate of cells was determined by an FITC Annexin V Apoptosis Detection Kit (BD Bioscience, New Jersey, USA). Using 5 μL FITC Annexin V and 5 μL PI (50 μg/mL) DNA labeling solution, the cells were incubated for 15 min. After staining, the cells were rinsed and then analyzed by using a BD Accuri™ C6 Flow Cytometer (BD Bioscience, New Jersey, USA). For each sample, the proportions of normal apoptosis (AV-, PI-), early apoptosis (AV+, PI-) and late apoptosis (AV+, PI+) were analyzed.

2.15. Measurement of intracellular reactive oxygen species (ROS)

The ROS level was measured through CM-H2DCFDA, a chloromethyl derivative of H2DCFDA (Life Technologies, Invitrogen, Carlsbad, USA). In brief, the cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. When the cells reached to 80% confluence, they were washed, incubated using 20 μmol/l CM-H2DCFDA at 37 °C for 30 min and then exposed to experimental conditions. The fluorescence emission spectra were then measured at an excitation wavelength of 495 nm and an emission wavelength of 525 nm (Victor 3 Multilabel Microplate Reader; PerkinElmer, Waltham, USA).

2.16. Measurement of mitochondrial membrane potential (MMP)

The MMP was measured by using a JC-1 assay kit (Jianchen, China). The OGD-treated HBMECs were rinsed with PBS solution (2000 rpm)

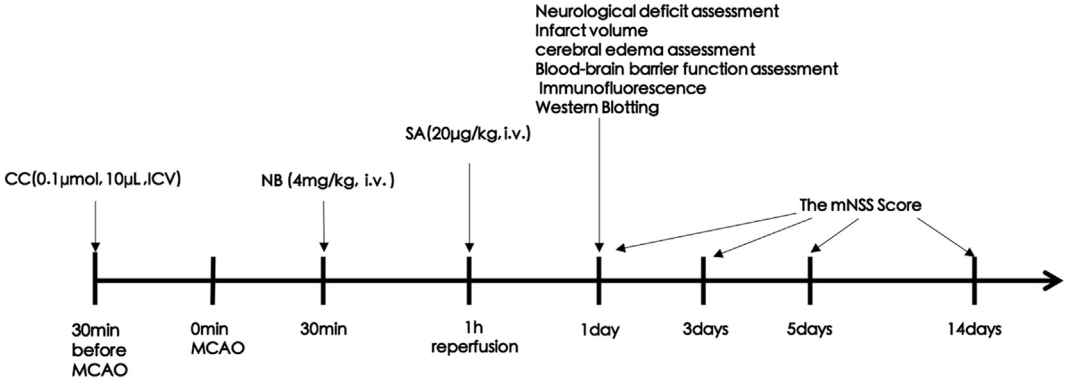

Fig. 1. Timeline of animal experimental procedures. MCAO: Middle Cerebral Artery Occlusion; mNSS: Modified neurological severity score; CC: compound C; NB: norbinaltorphimine; SA: Salvinorin A.

for 3 min and incubated using JC-1 reagent (500ul) for 30 min. JC-1 is a kind of lipophilic cationic dye that can enter mitochondria. When the MMP was high, it can form aggregates; and if not, it forms monomers. The value of JC-1 was measured with a flmo cytometer (BD Biosciences, USA). The collapse of MMP in OGD-treated cells was determined by reduced FL-2 fluorescence (red) and increased FL-1 fluorescence (green). The ratio of FL2/FL1 was analyzed using a flow cytometer.

2.17. Analysis of intracellular calcium concentration ($[Ca^{2+}]i$)

In brief, the cells preloaded with 5 μM of Fura-3 AM were dissolved in DMSO (Beyotime, Shanghai, China) for 1 h at room temperature. Images of the Fura-3-loaded cells were captured using a laser confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) and analyzed using Image-Pro Plus v6.0 software (Media Cybernetics, Crofton, MA, USA) at an excitation wavelength of 488 nm and emission wavelength of 525 nm. The gray value of the fluorescence represents $[Ca^{2+}]i$. The levels of $[Ca^{2+}]i$ were expressed as umol/g.

2.18. Measurements of intracellular ATP

The intracellular levels of ATP were measured with specific kits (Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions and then normalized to the protein concentrations, which were determined using the bicinchoninic acid method. The levels of ATP were expressed as umol/g.

2.19. Transmission electron microscopy (TEM)

To investigate the mitochondria, HBMECs were observed under a TEM. The samples were centrifuged at 1000 rpm, fixed in 1% glutaraldehyde in PBS at 4 °C for 3 h and then washed five times using 0.1 M cacodylate buffer containing 0.1% $CaCl_2$. After that, the samples were once again fixed using 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) that contained 0.1% $CaCl_2$ for 1 h. After rinsing with cold PBS, the cells were dehydrated through a graded series of ethanol and propylene oxide at 4 °C. After resin polymerization was performed at 55 °C for 24–36 h, serial sections were mounted on formvar-coated slot grids, and then the high resolution pictures were captured.

2.20. Western blotting

Twenty-four hours after MCAO, the cerebral cortex was fetched and homogenized. The protein was sampled and determined. The protein samples (40 μg/lane) were resolved in a 10% acrylamide gel for SDS-PAGE and then moved onto a polyvinylidene fluoride membrane (PVDF, Millipore). The PVDF membranes were then blocked using 5% non-fat dry milk in TBS with Tween 20 (TBST), incubated by rabbit antibodies, anti-AMPKα, 1:1000 (CST), rabbit, anti-phospho-AMPK, 1:1000 (CST), rabbit, anti-Mfn2 1:1000 (CST) and rabbit, anti-β-actin, 1:1000 (CST) at 4 °C for overnight and then washed with TBST. After that, the membrane was incubated with proper secondary antibodies (1:2000) at room temperature for 2 h and then washed with TBST. BioRad imaging system (BioRad, Hercules, USA) was used to visualize the protein band, and then quantified by using Quantity One analysis software (West Berkeley, California, USA).

2.21. Statistical analysis

The data were presented as mean ± SEM using GraphPad Prism 6.0 (Graphpad, San Diego, USA). The between group differences were evaluated by using one-way ANOVA and Tukey's range test. All the experiments were conducted at least six times.

3. Results

3.1. SA mitigated focal cerebral ischemia/reperfusion injury by AMPK in rats

Several scholars have demonstrated that KOR agonists can mitigate cerebral ischemic injury and protect neurological functions (Chen et al., 2016; Yang et al., 2011; Zhou et al., 2015). In the present study, SA, an efficient KOR agonist, was used to observe its effects on cerebral vessels and vascular endothelial cells. Cerebral ischemia often led to disordered blood-brain barrier function, high permeability of blood-brain barrier and cerebral edema and aggravated apoptosis, simultaneously causing short-term and long-term neurological damage (Fig. 1). From Fig. 2, the mNSS scores of the rats are decreased on days 1–14 after MCAO, which suggested that the neurological impairment of the rats caused by cerebral ischemia caused short-term and long-term behavioral disorder. SA treatment improved the mNSS score, and alleviated the brain damage after ischemia; however, NB and CC blocked the protection of SA. As shown in Fig. 3, all experimental groups showed a decrease in neurological scores when compared with the sham group, meaning that the MCAO models in the experimental groups are successfully established (Fig. 3B). TTC staining showed a large area infarction of brain tissue after ischemia. But intravenous administration of SA immediately after reperfusion reduced the infarct volume (Fig. 3C). The brain water content and the transparency of EB indicated that cerebral ischemia increased the brain water content, damaging the blood-brain barrier and augmenting the extravasation of EB. After SA treatment, the brain water content and cerebral edema were reduced effectively, the extravasation of EB was brought down and the blood-brain barrier function was also improved (Fig. 3D, E).

Numerous studies have shown that regulation of AMPK activation plays a crucial role in the maintenance of cell function (Ashabi et al.,

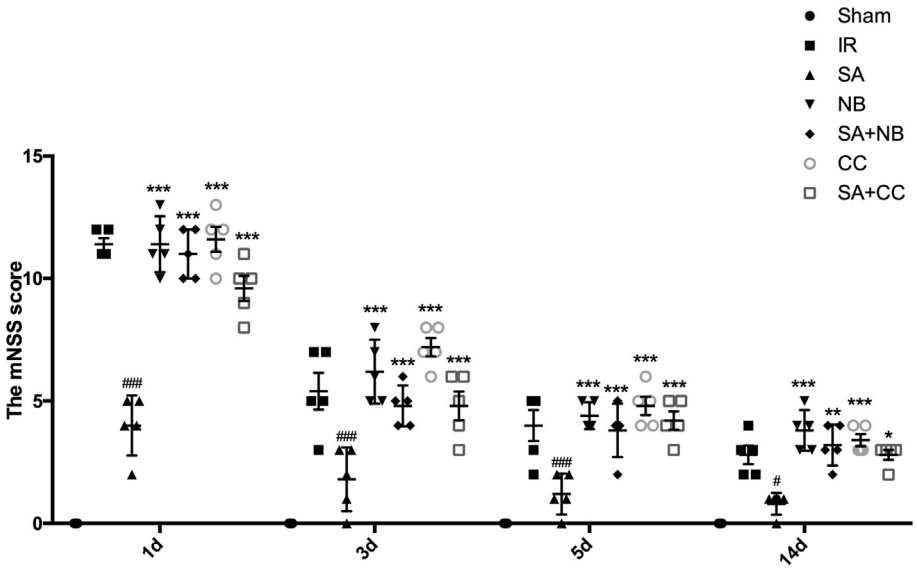

Fig. 2. SA mitigates short-term and long-term behavioral disorders of ischemic stroke. The assessment of modified neurological severity score (mNSS) after MCAO (1, 3, 5, and 14 days). Data in the bar graphs are presented as mean ± SEM for $n = 5$.$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, compared with the SA group. $^{\#\#\#}P < .001$, compared to the MCAO group.

2014; Cao et al., 2017; Jiang et al., 2018). After activation and stimulation of cells, AMPK can mitigate oxidative stress, inflammatory response, *etc.* to protect the endothelial cells. Our previous studies have also supported the key role of AMPK in alleviating cerebral ischemia/reperfusion injury. So, in the present study, the effect of AMPK on the protection of cerebral vessels by SA was discussed. The levels of total AMPK and pAMPK in ischemic cortex 24 h after MCAO were determined by western blotting. The results revealed no significant differences among the groups in the expression level of total AMPK. Cerebral ischemia inhibited the phosphorylation of AMPK, while SA

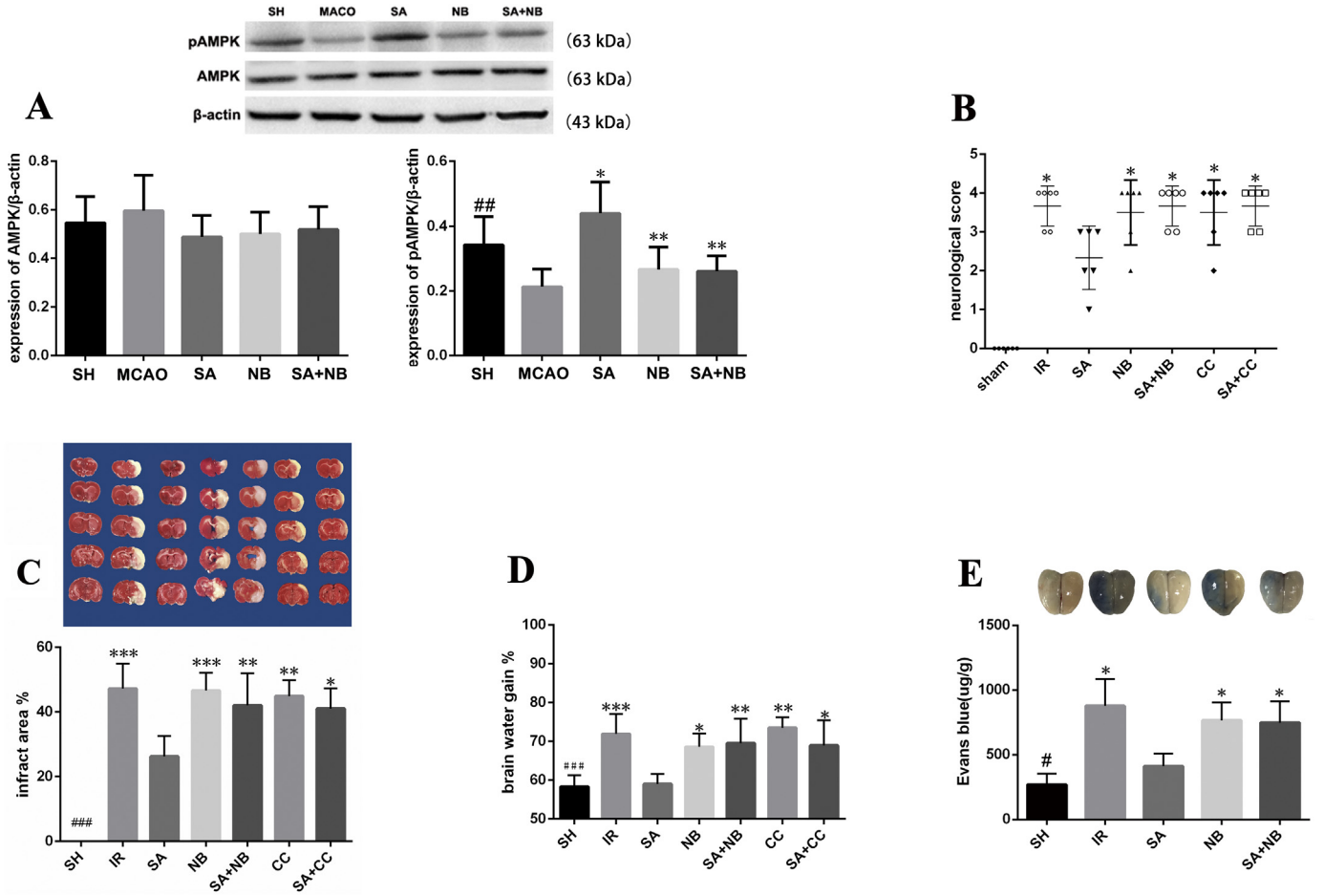

Fig. 3. SA mitigated focal cerebral injury by up-regulating the levels of pAMPK after ischemia. (A) Western blotting of AMPK and pAMPK expression in the ischemic cortex. β-Actin was used as the load control. (B–E) The neurological scores, the TTC staining test of brain tissues, the percentage of brain water gain and the transparency of EB of the rats measured for 24 h after MCAO. Data in the bar graphs are presented as mean ± SEM for $n = 6$.$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, compared with the SA group. $^{\#\#\#}P < .001$, compared to the MCAO group.

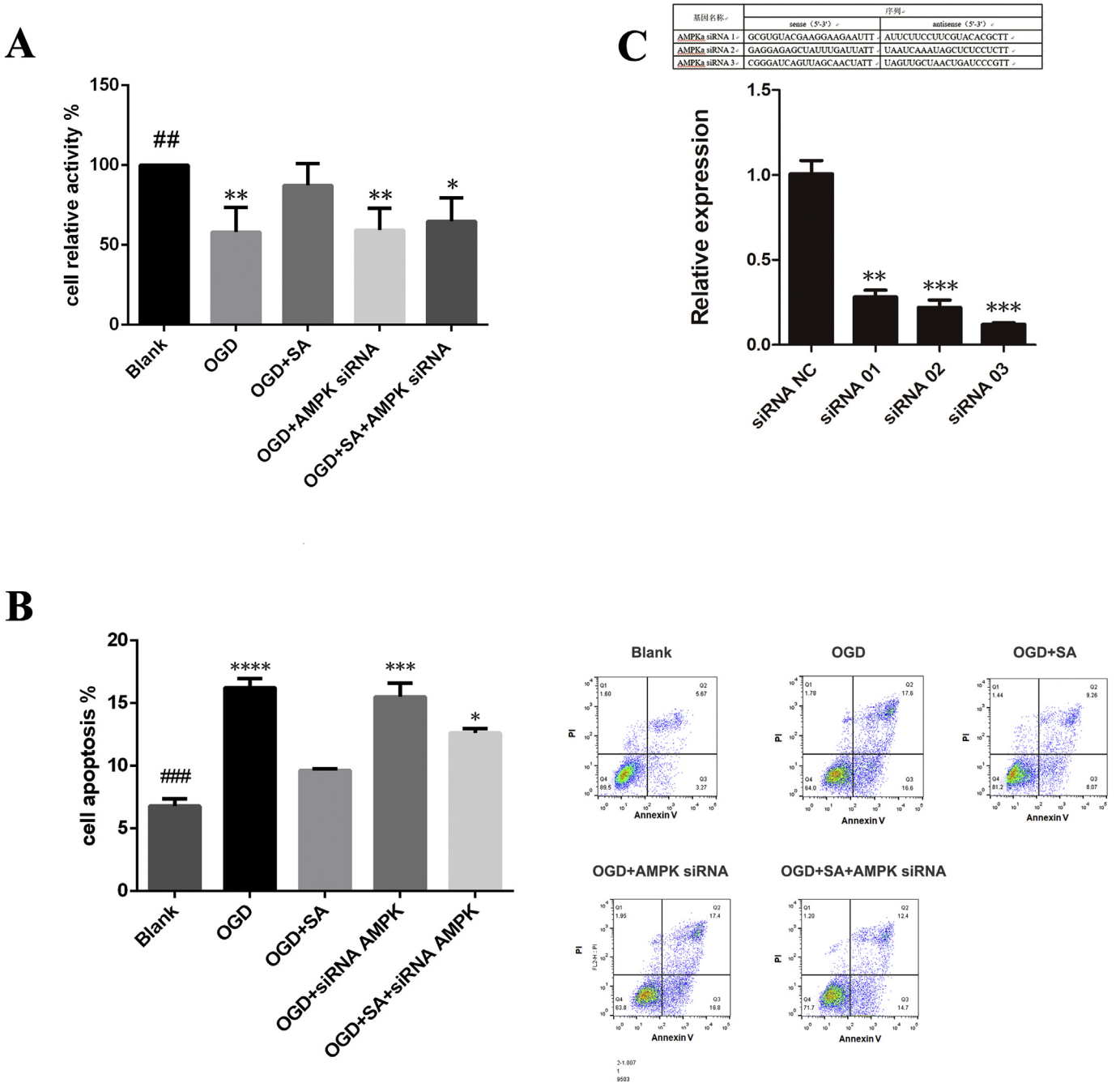

Fig. 4. SA can restore the viability and mitigate apoptosis of endothelial cells by activating AMPK after OGD injury. (A) CCK-8 analysis of the relative activity of HBMECs after OGD treatment. (B) Flow-cytometry analysis of HBMECs apoptosis after OGD treatment. Data in the bar graphs are presented as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .001$, compared to the OGD + SA group. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, compared to the OGD group. (C) RT-PCR analysis of mRNA expression of AMPK.$^{**}P < .01$, $^{***}P < .001$, compared to the NC group.

treatment immediately after reperfusion significantly up-regulated the levels of pAMPK (Fig. 3A). To explore the role of AMPK in protection by SA, CC, an AMPK blocker, was injected through the lateral ventricle of rats to block the effect of AMPK. The results showed that after injecting CC, the neurological score of the rats was dropped sharply (Fig. 3B), the infarct volume was expanded (Fig. 3C) and the brain water content grew (Fig. 3D) when compared with SA. This suggested that SA treatment induced a protective effect on cerebral ischemia injury by activating AMPK. To ascertain SA as an effective target, NB (2 mg/kg), a KOR antagonist, was used to block KOR. Basically, the effect of SA was counteracted, indicating that SA induced an effect by activating KOR.

3.2. SA mitigated OGD injury in HBMECs

Our previous studies showed that SA can protect the functions of cerebral vessels after ischemia. To further investigate the role SA in cerebrovascular endothelial cells, the cerebrovascular endothelial cells were exposed to OGD conditions. As shown in Fig. 4, the HBMECs were deprived of oxygen for 6 h and then were reoxygenated for 24 h. Using CCK-8, the viability of cells was measured. The results revealed that after OGD treatment, the viability of endothelial cells was significantly inhibited. The flow cytometer also showed that the apoptosis was aggravated. When HBMECs were reoxygenated, the addition of SA to the medium significantly restored the viability of cells (Fig. 4A) and

mitigated apoptosis after OGD treatment (Fig. 4B). In this study, AMPKα siRNA transfected cells were used to investigate the role of AMPK. The efficiency of siRNA transfection was assessed by analyzing the mRNA expression of AMPK (RT-PCR), (Fig. 4C). The results showed that siRNA 01, siRNA 02 and siRNA 03 significantly reduced the expression of AMPK, and the effect of siRNA 03 interference was considered as the most significant. So, siRNA 03 was selected for subsequent experimental studies. After interfering the function of AMPK, the effect of SA on the viability of endothelial cells and apoptosis were counteracted (Fig. 4B).

3.3. SA improves mitochondrial function in HBMECs

Through the above research, we confirmed that SA can activate AMPK and effectively protect cerebrovascular endothelial cells injured by cerebral ischemia. Some scholars have reported that AMPK was connected with mitochondrial function, and so the effect of SA on the mitochondria of endothelial cells was discussed. Using DCFH-DA, a fluorescence probe, the mitochondrial function was measured. The ROS level of HBMECs showed that the ROS level in the cells showed a significant growth after OGD treatment. While after SA intervention, the ROS level in the cells was decreased quickly (Fig. 5A). Furthermore, the morphology of mitochondria was observed by TEM. After OGD treatment, the mitochondria were swollen, the mitochondrial cristae fracture disappeared, vacuolation and other lesions were alleviated. During reoxygenation and after SA treatment, the morphology of mitochondria was significantly improved, the volume has returned to normal and the mitochondrial cristae were rapidly grown (Fig. 5B). The normality of MMP was considered as a prerequisite for maintaining oxidative phosphorylation of mitochondria and ATP production and a necessity for maintaining the mitochondrial function. An important change caused by mitochondrial impairment was the collapse of MMP, and so the changes of MMP were observed. JC-1 test revealed that the MMP of HBMECs was decreased sharply after OGD treatment. During reoxygenation, the level of MMP was restored after administration of SA (Fig. 5C). As shown in Fig. 5D, The results showed that after OGD treatment, $[Ca^{2+}]i$ was higher than that in the control group, suggesting the HBMECs were overloaded with Ca^{2+}. When SA was added, $[Ca^{2+}]i$ was down-regulated to near normal levels. When interfered by si-AMPK, the down-regulation of $[Ca^{2+}]i$ by SA was blocked, and $[Ca^{2+}]i$ re-emerged. Treatment with OGD decreased the intracellular ATP content of HMEBCs, and the cells showed a marked lack of cellular energy. After treatment with SA before reoxygenation, intracellular ATP levels were increased when compared with OGD group, and cell energy metabolism tended to be normal. After interference with si-AMPK and blockage of AMPK function, the effect of SA was antagonized, decreasing the intracellular ATP content, and inhibiting the energy metabolism of HBMECs (Fig. 5E). The results showed that SA effectively mitigated the damage of mitochondria in HBMECs caused by OGD and improved mitochondrial functions. Using siRNA interference, the function of AMPK was blocked and the results revealed that the protective effects of SA on morphology and function of mitochondria were counteracted, proving that AMPK activation was an important step for SA to regulate mitochondrial function in endothelial cells (Fig. 5).

3.4. SA regulates the expression of Mfn2 both in vitro and in vivo through AMPK pathway

Mfn2 is a key protein in mitochondria that controls mitochondrial fusion. It plays an important role in mitochondrial function maintenance. To confirm the regulatory mechanism of SA in mitochondria, the effect of SA on Mfn2 was observed. The results of western blotting showed that in MCAO rat models, ischemic injury significantly increased the expression level of Mfn2. By injecting SA intravenously immediately after reperfusion, the protein content of Mfn2 was increased rapidly (Fig. 6A).

In *in vivo* experiment, the results of western blotting showed that after MCAO treatment, the expression of Mfn2 in cerebrovascular endothelial cells was decreased. The addition of SA after 24 h increased the expression level of Mfn2. CC was added before MCAO and the results showed that the effect of SA on the expression of Mfn2 in HBMECs was inhibited, proving that the AMPK pathway mediated the promotion of Mfn2 in cerebrovascular endothelial cells by SA (Fig. 6B).

We marked Mfn2 (green) and CD31 (red) using immunofluorescence to clarify the expression of Mfn2 in cerebrovascular endothelial cells. The results showed that the cerebrovascular endothelial cells (green spots) in the cerebral cortex of rats were significantly decreased after ischemia, suggesting that the expression of Mfn2 was down-regulated. After SA treatment, the green spots on cerebrovascular endothelial cells were significantly increased. The expression of Mfn2 in cerebrovascular endothelial cells was up-regulated (Fig. 6C). Before SA treatment, the AMPK antagonist CC was injected through lateral ventricle, and the green spots on endothelial cells were significantly decreased, suggesting that CC significantly inhibited the increased expression of Mfn2 and the AMPK pathway involvement in the regulation of Mfn2 expression in the mitochondria by SA.

4. Discussion

Ischemic stroke is a process where in the blood flow is blocked in the blood supply area, the metabolism of brain cells has become disordered, and the oxidative stress, inflammatory response and other pathological processes occur in the cells, finally damaging the cranial nerves and leading to disability and even death at times. At present, the incidence of ischemic stroke remained very high. Tissue plasminogen activator (tPA) is the only effective drug approved by the FDA for the treatment of acute ischemic stroke, but its clinical applications are restricted due to the side effects and therapeutic time window (Yang and Mu, 2017). Therefore, studies on ischemic stroke are still warranted.

Cerebrovascular endothelial cells are the most important components of cerebral vessels and also the most vulnerable cells in cerebral ischemia. The normality of their function is guaranteed due to their functions in blood-brain barrier and cerebral vessels. However, the studies on cerebrovascular endothelial cells are still inadequate. In the present study, we explored the protection mechanism of SA, a KOR agonist, in cerebrovascular endothelial cells after ischemia/reperfusion at cellular level and in animals. The following findings were observed: SA effectively protected cerebral vessels after ischemia/reperfusion, reducing the damage to the blood-brain barrier and protecting the neurological functions after ischemia. SA dramatically mitigated the damage and apoptosis of HBMECs caused by OGD. Secondly, SA activated AMPK in the MCAO model of rats. By blocking the function of AMPK by using a blocker and siRNA technology, the protective effect of SA was antagonized. After that, by studying the mitochondrial functions, SA activated AMPK, promoted the expression of Mfn2, reduced the outbreak of ROS, maintained the morphology of mitochondria, stabilized the MMP and protected mitochondrial functions in cerebrovascular endothelial cells. By activating AMPK, it was clarified that SA protects the morphology and functions of mitochondria in cerebrovascular endothelial cells after cerebral ischemia, reducing the degree of oxidative stress in endothelial cells, diminishing the damage and apoptosis of cells and reducing the damage to the blood-brain barrier.

Our previous studies have shown that SA dramatically reduced cerebral edema caused by cerebral ischemia (Xin et al., 2016), and so SA was considered to have some protective effects on cerebral vessels. In the present study, we verified this hypothesis on MCAO model and the results showed that immediate administration of SA after ischemia/reperfusion significantly reduced cerebral edema 24 h after ischemia and distinctively improved the permeability of blood-brain barrier after ischemia. Meanwhile, the effect of SA was blocked by a KOR antagonist, indicating that SA produced an effect by activating KOR receptor. This

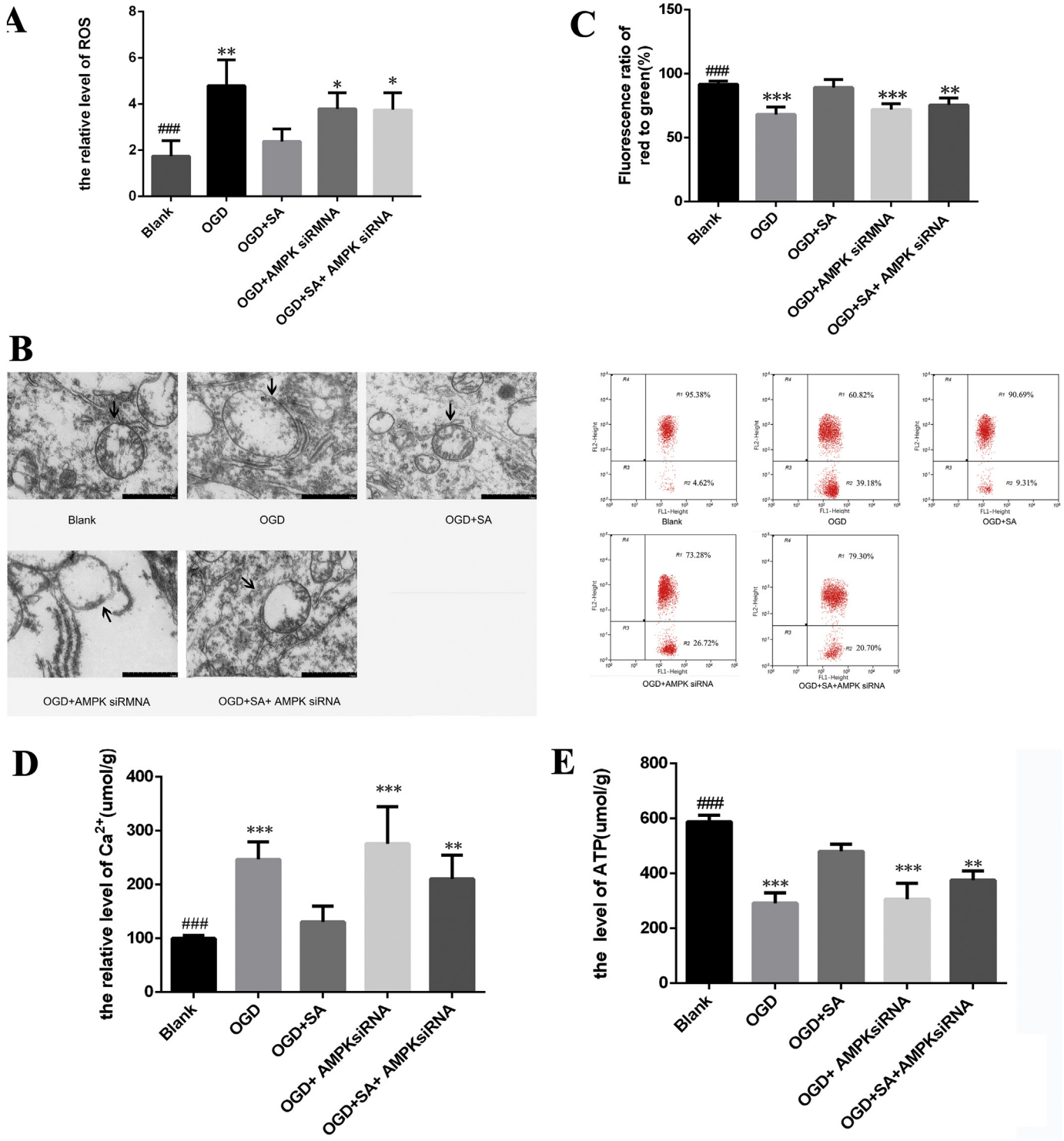

Fig. 5. SA preserves mitochondrial morphology and function in HBMECs through AMPK pathway after OGD injury. (A) Measurement of ROS level in HBMECs after OGD treatment. (B) Observation of the morphology of mitochondria by TEM. (C) JC-1 test of MMP in HBMECs after OGD treatment. (D) Measurement of $[Ca^{2+}]i$ in HBMECs after OGD treatment. (E) Measurement of ATP level in HBMECs after OGD treatment. Data in the bar graphs are presented as mean ± SEM.$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, compared with the OGD + SA group. $^{\#\#\#}P < .001$, compared to the OGD group.

was in consistent with the findings by other scholars. KOR agonists improved cerebral edema after cerebral ischemia (Yang et al., 20151, Yang et al., 2015), and protected the integrity of blood-brain barrier (Chen et al., 2016; Xi et al., 2017; Cao et al., 2017; Chunhua et al., 2014). Our previous studies have also shown that SA can protect the self-regulation of cerebral blood flow after cerebral ischemia through PI3K/Akt/cGMP signaling pathway (Dong et al., 2018; Wang et al., 2012) All these effects were inseparable from cerebrovascular endothelial cells. However, so far, the regulatory mechanism of SA in the functioning of endothelial cells remained unclear. So, in this study, we investigated by using a HBMEC OGD model and found that by activating KOR, the intervention of SA had a protective effect on cerebrovascular endothelial cells and mitigated the apoptosis and improved the vitality of HBMECs caused by OGD treatment. The effect of SA on

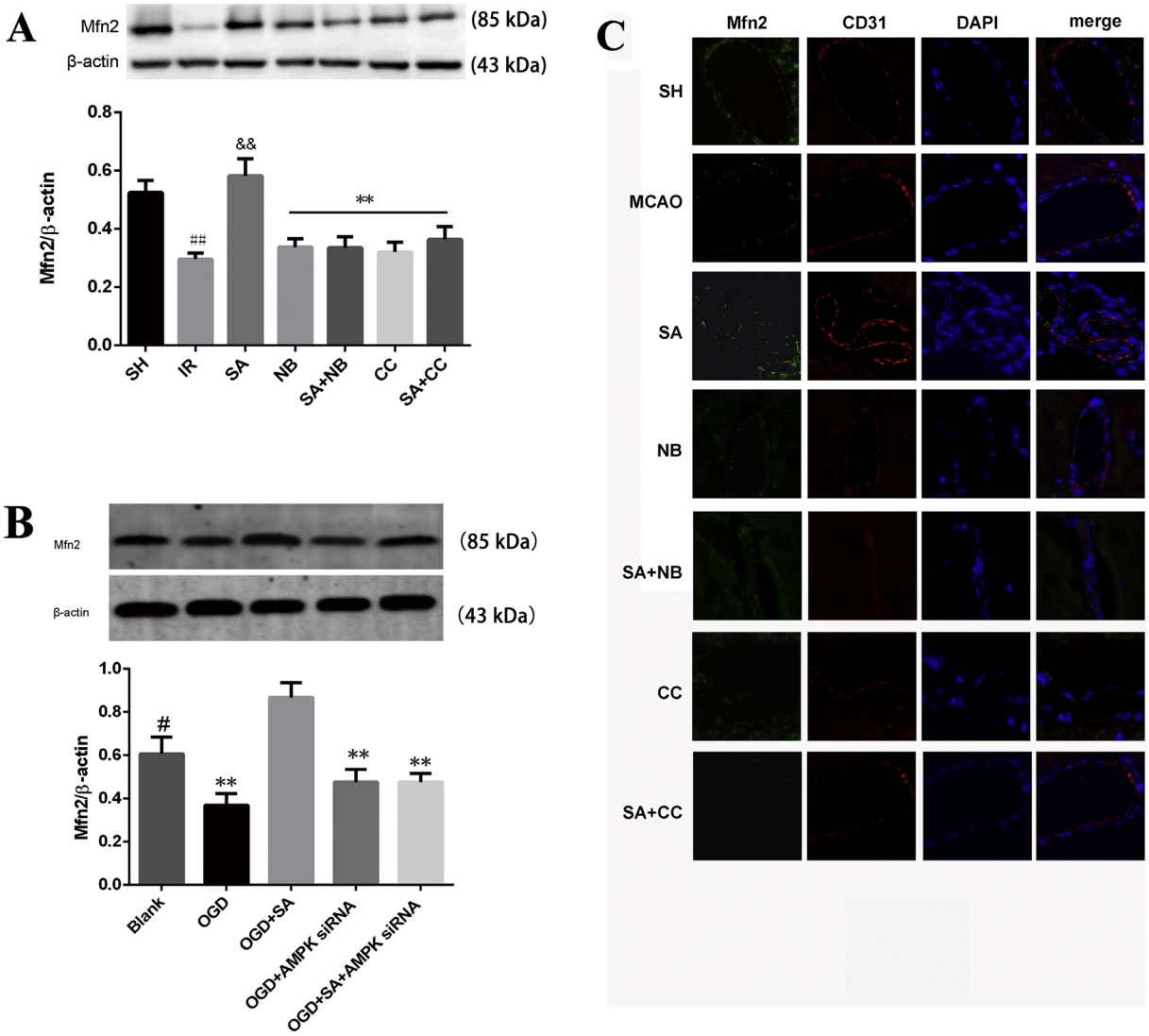

Fig. 6. SA up-regulated the expression of Mfn2 by activating of AMPK in ischemic stroke. (A) Western blotting of Mfn2 protein in the ischemic cortex of rats. β-actin was used as the load control. Data in the bar graphs are presented as mean ± SEM for $n = 6$. $^{**}P < .01$, compared with the SA group. $^{\#\#}P < .01$, compared to the SH group.$^{\&\&}P < .01$, compared to the MCAO group. (B) Western blotting of Mfn2 protein in HBMECs after OGD injury. β-actin was used as the load control. Data in the bar graphs was presented as mean ± SEM. $^{**}P < .01$, compared with the OGD + SA group. $^{\#}P < .05$, compared to the OGD group. (C) Immunofluorescence analysis of Mfn2 (green) and CD31 (red) expression in cerebrovascular endothelial cells. Nuclei were stained with DAPI (magnification, × 400). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cerebrovascular endothelial cells was confirmed.

Ischemia/reperfusion can lead to disordered energy metabolism and mitochondrial dysfunction in cerebrovascular endothelial cells. This is the most important cause of cell damage and can lead to the disruption of blood-brain barrier and cerebral edema. Ischemia and hypoxia can lead to disorders of mitochondrial function, increased production of cellular ROS, and induced cellular energy disorders, imbalance of metabolites and ion transport. Mitochondrial store Ca^{2+} controls the homeostasis of calcium concentration in cells. When mitochondrial function is impaired, it causes Ca^{2+} dysfunction, leading to intracellular Ca^{2+} overload, and eruption of intracellular calcium ion content (Moller et al., 2017; Karam et al., 2017). Mitochondrial mites contain ATPases, a site in which ATP is synthesized. When mitochondrial function is impaired, intracellular ATP synthesis is down-regulated (Xue et al., 2019; Hayakawa et al., 2018). Therefore, we observed the effect of SA on mitochondrial function after OGD in HBMECs by observing $[Ca^{2+}]i$ and ATP levels. As an energy manager of cells, mitochondria are characterized by their ability to achieve biological functions by highly-coordinated fission and fusion (Ruthel and Hollenbeck, 2003). Mitochondria not only play an essential role in energy production, but are also involved in the regulation of apoptosis, autophagy and necroptosis. More and more evidences have showed that maintaining the mitochondrial functions is important for the survival of neurons and improvement of nervous system functions (Huang et al., 2016). Among all cerebrovascular units, the content of mitochondria in endothelial cells remained higher when compared to those in other cells, suggesting that mitochondria play a more significant role in endothelial cells. Numerous studies have proved that ischemia/reperfusion injury can lead to apoptosis and necrosis of neurons by inducing mitochondrial DNA damage, energy disturbances, mitochondrial fragmentation and oxidative stress (Chun et al., 2018). Also several studies have shown that mitochondrial fission and fusion balance the mitochondrial functions in a dynamic way. Under a damaging stress, the mitochondrial fission is intensified and the mitochondrial fusion is inhibited, leading to mitochondrial fragmentation. After MCAO model establishment, the mitochondrial fission occurred after 3 h of reperfusion (Liu et al., 2018a, 2018b).

The role of mitochondria in endothelial cells has also been gradually

emphasized, and mitochondria play a crucial role in maintaining the normal function of endothelial cells during brain trauma or cerebral ischemic injury. (Nguyen et al., 2018; Chitturi et al., 2019; Jiang et al., 2018). Although we and other scholars have found that KOR agonists play a significant protective role in cerebral ischemic injuries, the regulatory effect of KOR agonists on mitochondria has not yet been studied. Hence, in the present study, HBMECs were used. TEM showed that the mitochondria were swollen and the mitochondrial cristae were broken and vacuolated after OGD treatment. After SA treatment, the morphology of mitochondria was significantly improved and returned to normal. We also used a JC-1 fluorescence probe to measure the changes in MMP. The results showed that after OGD treatment, the MMP was decreased observably, suggesting that the mitochondrial functions were disordered and an early apoptosis of cells occurred (Wang et al., 2018b; Kang et al., 2018). After SA intervention, the MMP was returned to normal. The normal functions of mitochondria were maintained. The damage of mitochondria may lead to oxidative stress in cells and increased the release of ROS (Bombaça et al., 2018). In this study, the ROS level in HBMECs was dramatically increased after OGD treatment. After SA treatment, the ROS level of cells was reduced effectively, and the damage of oxidative stress to cells was mitigated. The experimental findings of endothelial cells were consistent with the phenomena observed in *in vivo* animal model, indicating that the KOR agonist SA protects the mitochondrial functions in cerebrovascular endothelial cells after ischemia/reperfusion injury, and dramatically reduces the oxidative stress and apoptosis of cells and the degree of cerebral trauma. We further explored the protection mechanism of SA, which had the highest potential for clinical applications on ischemia/reperfusion injury.

After the occurrence of cerebral ischemia, due to lower blood supply, the energy balance was imbalanced and the synthesis of adenosine triphosphate (ATP) was disturbed. When the ratios of ADP/ATP and AMP/ATP were increased in a low energy state, AMPK is activated to stabilize the energy in the cells (Lewinska et al., 2017). More and more studies have shown that abnormal energy metabolism after ischemia/reperfusion injury acts as a key factor for local inflammatory response and other pathological processes. In our previous studies, the activation of AMPK regulated the disorder of energy metabolism in cells, playing an anti-inflammatory role after ischemia/reperfusion injury and protecting the neurons (Wang et al., 2018b). AMPK exerts various regulatory mechanisms during ischemic stroke, including oxidative stress, autophagy, apoptosis, mitochondrial dysfunction, glutamate excitotoxicity, neuroinflammation, and angiogenesis (Jiang et al., 2018). Previous studies have shown that activation of AMPK can reduce the damage of mitochondria, and inhibit oxidative stress and apoptosis (Wang et al., 2018b). Especially after ischemia/reperfusion injury, the activation of AMPK (Thr172) can regulate the mitochondrial functions (Liu et al., 2017; Li et al., 2017b). In this study, administration of SA immediately after reperfusion in an MCAO model significantly promoted the phosphorylation level of AMPK in the brain of rats. CC (an AMPK blocker) treatment impaired the effect of SA on the improvement of cerebral infarction and cerebral edema, as well as the effect on the improvement of cerebral functions. In HBMEC OGD model, we also validated that under the interference of siRNA, the effect of SA on higher vitality and lower apoptosis of endothelial cells was weakened, suggesting that AMPK also played a key role in the protection of cerebrovascular endothelial cells by SA.

We have verified that SA can protect mitochondria in vascular endothelial cells after cerebral ischemia. To figure out whether AMPK was involved in this process, further study was carried out. The results showed that in the HBMEC OGD model, before OGD treatment, after siRNA blocking of AMPK, endothelial cells showed mitochondrial dysfunction even after SA treatment. It was observed under TEM that the mitochondrial cristae were broken and vacuolated, the MMP was dropped significantly and the content of ROS was significantly increased, suggesting that SA treatment protected mitochondrial functions in the endothelial cells through AMPK.

Mfn2 exists in the membrane of mitochondria, and activation of Mfn2 promotes mitochondrial fusion and significantly reduces the dysfunction caused by mitochondrial fragmentation (Schrepfer and Scorrano, 2016). Mitochondrial fusion and subcellular transport depends on two mitochondrial fusion proteins, Mfn1 and Mfn2. The activation of Mfn2 reverses the damage of mitochondria (Rocha et al., 2018; Enzmann et al., 2018). After cerebral ischemia, the expression of Mfn2 was dramatically decreased and the homeostasis of Ca2 + in the mitochondria was disrupted, leading to mitochondrial fusion and functional disorders (Peng et al., 2015). Mfn2 activation reduced autophagy of mitochondria, degraded the damaged mitochondria and interrupted the transduction of damage signal of mitochondria. Mfn2 not only plays a role in mitochondrial fusion, but is also involved in the regulation of cell proliferation, oxygen metabolism, cell autophagy, the antiviral signaling of mitochondria and unfolded protein responses (Chun et al., 2018; Sebastián et al., 2016). Both *in vivo* and *in vitro* results showed that the expression of Mfn2 was decreased significantly after ischemia and hypoxia (Wang et al., 2018a; Peng et al., 2018; Ge et al., 2018; Zhang and Yu, 2018), leading to the disruption of homeostasis of Ca^{2+} in mitochondria and disorder of mitochondrial fusion and functions (Peng et al., 2015). The low expression of Mfn2 was closely related to the upsurge of oxidative stress and the occurrence of apoptosis after hypoxic-ischemic damage of cells (McLaughlin et al., 2017; Liu et al., 2017; Cao et al., 2017). Research showed that mitochondrial fragmentation occurs 3 h after ischemia and peaks within 24 h (Lückl et al., 2018). Similar results were obtained in our study. The expression of Mfn2 in vascular endothelial cells was significantly down-regulated 24 h after MCAO establishment and OGD treatment. After SA intervention, the expression level of Mfn2 in the brain tissues was dramatically increased. Immunohistochemistry marker test of CD31 and Mfn2 showed that the expression of Mfn2 in cerebrovascular endothelial cells was dramatically increased. On the other hand, it also demonstrated that SA effectively promoted the expression of Mfn2 in cells 24 h after hypoxic-ischemic stimulation in the HBMEC OGD model. To clarify the role of AMPK in the regulation of Mfn2 expression by SA, we injected CC through the lateral ventricle to antagonize AMPK and discovered that the expression of Mfn2 in the brain tissues was dropped remarkably. Immunofluorescence test showed that the fluorescence marker of Mfn2 in endothelial cells became weak. Similar results were obtained in HBMECs. After siRNA blockage of AMPK, the up-regulation of Mfn2 in endothelial cells by SA was effectively inhibited, indicating that AMPK was involved in the regulation of Mfn2 by SA after cerebral ischemia. The present study illustrated on how SA protects cerebrovascular endothelial cells by regulating the mitochondrial functions. However, the mitochondria are organelles with dynamic balance between mitochondrial fission and fusion. We mainly observed the role of mitochondria, but the influence on the balance of mitochondrial functions warranted further investigation.

In conclusion, SA immediately after reperfusion reduced the damage and apoptosis of endothelial cells after cerebral ischemia effectively, decreased the permeability of blood-brain barrier and mitigated cerebral edema. SA reduced mitochondrial deformation in endothelial cells, stabilized MMP, reduced the outbreak of ROS, up-regulated the expression of Mfn2 and ultimately protected the mitochondrial functions. The activation of AMPK remains critical in protecting SA in the mitochondria of endothelial cells after cerebral ischemia.

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Availability of data and materials

All data generated or analyzed during this study are included in the

manuscriptsubmission files.

Authors' contributions

Wang and He designed the experiments. Dong and Zhou performed the majority ofthe experiments with the help of Shi and Yao. Dong, Zhou and Xinperformedthe error analysis. Wang and He participated in the discussion about the resultsand in the manuscript preparation. Dong and Wang wrote themanuscript. All authors readand approved the final manuscript.

Ethics approval

Animal protocols were approved by the Animal Care and ExperimentalCommittee of the School of Medicine of Shanghai Jiao Tong University.

Declaration of competing interests

The authors declare that they have no competing interests.

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