Astragaloside IV Protects Primary Cerebral Cortical Neurons from Oxygen and Glucose Deprivation/Reoxygenation by Activating the PKA/CREB Pathway

Bingjie Xue, a,† Jisheng Huang, b,† Bo Ma, a Bin Yang, a Dennis Chang c and Jianxun Liu a,c,*

a Institute of Basic Medical Sciences, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing, China
b Tianjin Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China
*NICM Health Research Institute, Western Sydney University, Locked Bag 1797, Penrith, NSW 2751, Australia

Abstract—Stroke is one of the major leading causes of death and disability worldwide, and post-stroke cognitive impairment is a major contributor to this disability. Astragaloside IV (AST-IV) is a primary bioactive compound of Radix Astragali, which is widely used in traditional Chinese medicine to treat stroke. AST-IV was found to possess cognition-enhancing properties against ischemic stroke; however, the mechanisms underlying this effect remain largely elusive. Mitochondrial health is critical to cell viability after ischemic injury. Cyclic AMP response element-binding protein (CREB) is a transcription factor that can be activated by protein kinase A (PKA) to preserve mitochondria, regulate memory and cognitive functions. We used an in vitro model of ischemic injury via oxygen and glucose deprivation (OGD) of cultured neurons, which led to PKA inactivation and decreased CREB phosphorylation, reduced cell viability, and increased neuronal apoptosis. We hypothesized that AST-IV could protect OGD-exposed cerebral cortical neurons by modulating the PKA/CREB signaling pathway and preserving mitochondrial function. We found that the mitochondrial and cellular injuries induced by OGD were reversed following treatment with AST-IV. The activity of neuronal mitochondria was evaluated by measuring the mitochondrial potential and the levels of reactive oxygen species (ROS) and adenosine triphosphate (ATP). AST-IV significantly enhanced PKA and CREB phosphorylation and prevented OGD-induced mitochondrial dysfunction, thereby protecting neurons exposed to OGD from injury and death. Furthermore, the effects of AST-IV were partially blocked by a PKA inhibitor. Collectively, these data elucidated the molecular mechanisms underlying the protective effects of AST-IV against ischemic injury in cortical neurons. © 2019 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Key words: astragalus, ischemic stroke, mitochondria, ROS, protein kinase a, cyclic AMP response element-binding protein.

INTRODUCTION

Radix Astragali is an important herb used in traditional Chinese medicine for the treatment of cerebrovascular diseases such as stroke. It has been well documented that Radix Astragali or its bioactive components could ameliorate cognitive impairments induced by stroke (Tohda et al., 2010; Dun et al., 2016; Huang et al., 2017). Radix Astragali is one ingredient of a complex traditional herbal formula called the Buyanghuanwu decoction, which has been used in China to treat stroke and stroke-related diseases since the 17th century (Chen et al., 2007; Zhao et al., 2010; Liu et al., 2011). Astragaloside IV (AST-IV), a key bioactive component of Radix Astragali, has attracted increasing attention in recent years due to its potential therapeutic benefits for stroke-related cognitive impairment (Kim et al., 2015; Li et al., 2017). Here, we investigated the protective effects of AST-IV in cortical neurons subjected to oxygen and glucose deprivation (OGD; in vitro model of ischemic injury) and explored the possible mechanisms underlying the action of AST-IV.

Serine/threonine protein kinases are highly expressed in the central nervous system and are severely altered in response to ischemic insults (Domanska-Janik, 1996). Reduced phosphorylation of protein kinase A (PKA) was shown to play a critical role in the progression of post-ischemic brain injury (Zhang et al., 2017). Cyclic AMP response element-binding...
protein (CREB), a transcription factor activated by PKA, has been shown to regulate transcription in neurogenesis and was also implicated in cerebral ischemia (Kitagawa, 2007; Schölzke and Schwanger, 2007). CREB regulates a variety of intracellular signaling events, including neuronal growth, proliferation, synaptic efficacy, and long-lasting changes in synaptic plasticity, which affects the processes of memory and cognition (Paramanik and Thakur, 2013; Saura and Cardinaux, 2017; Yu et al., 2017). It has been found to exist and act both in the nucleus and the mitochondrial matrix (Ryu et al., 2005). Aberrant energy metabolism following cerebral ischemia induces mitochondrial impairments leading to reduced respiration, excessive production of reactive oxygen species (ROS), and depletion of adenosine triphosphate (ATP) (Fiskum et al., 1999; Perez-Pinzon, 2004). Therefore, maintaining mitochondrial health is critical in promoting ischemic tolerance by enhancing neuronal viability following ischemic injury (Perez-Pinzon et al., 2012).

Given that AST-IV protected the ischemic brain via a ROS-dependent mechanism (Lu et al., 2015; Wang et al., 2016), and that mitochondria was reported as the main source of ROS regulated by PKA/CREB (Zorov et al., 2006), we hypothesized that AST-IV could modulate the PKA/CREB pathway to preserve healthy mitochondrial function and thereby protect neurons from injury during ischemia. We revealed that AST-IV treatment led to the activation of PKA, which triggered CREB phosphorylation and prevented mitochondrial dysfunction in primary cortical neurons, thereby protecting them from OGD-induced neuronal injury and death.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**

The chemical reagents and kits used in this study are listed here: AST-IV (National Institutes for Food and Drug Control, China, #84687–43-4), H-89 (Sigma-Aldrich, USA, #81427), Hoechst 33342 (Invitrogen, USA, #H1399), Mitosox Red (Life Technologies, USA, #M36008), JC-1 dye (Life Technologies, USA, #T3168), Cell Counting Kit 8 (CCK-8) kit (DOJINDO, Japan, #CK04), Cytotoxicity Detection Kit (LDH) (Roche, Switzerland, #11644793001), and ATPlite Luminescence ATP Detection Assay system (PerkinElmer, USA, #6016941), B27 (Gibco, USA, #17504044), primary neuron basal medium (Gibco, USA, #A3582901), low-glucose Hank’s balanced salt solution (HBSS) (Solarbio, China, #H1025).

The antibodies used in this study are listed here: anti-PKA (Abcam, #ab187515), anti-phosphorylated (p-) PKA (p-PKA [T197]) (Abcam, #ab75991), anti-CREB (Abcam, #ab32515), anti-p-CREB (Abcam, #ab32096), anti-caspase-3 (Abcam, #ab4051), anti-cleaved caspase-3 (Abcam, #ab49822), anti-β-actin (Abcam, #ab6267), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam, #ab205718), anti-MAP2 (CST, #4542S), and anti-rabbit IgG (H + L), F(ab’)2 Fragment (Alexa Fluor-594 Conjugate) (CST, #8889).

**Fetal Cerebral Cortical Neuron Culture**

Cortical neuronal cultures were prepared as previously described (Xiaodi et al., 2016). Adult, pregnant Sprague-Dawley rats were supplied by the Experimental Animal Center of the Xiyuan Hospital (Beijing, China, License number: SCXK [Beijing] 2016–0002). All experimental procedures were carried out according to the protocol approved by the Ethics Committee for Animal Experimentation of Xiyuan Hospital and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23; revised 1996) and the UK Animals (Scientific Procedures) Act 1986.

Pregnant rats were euthanized using carbon dioxide at embryonic day 18. The fetuses were collected in ice-cold HBSS supplemented with 0.5% penicillin/streptomycin (P/S). They were decapitated and the whole brain was transferred into fresh, cold Dulbecco’s modified Eagle medium (DMEM)/F12 with 0.5% P/S. The isolated cerebral cortices were minced and incubated at 37 °C for 20 min in a solution of 0.125% trypsin. The tissue was washed in DMEM/F12 containing 10% fetal bovine serum and then passed through a 200-mesh filter screen. Following 15 min of centrifugation at 800 g and 4 °C, the pellet was resuspended in 4 mL Primary Neuron Basal Medium supplemented with 2% B27, 0.5% P/S, and Glutamax. Next, the cells were plated at a density of 1 × 10^5 cells/mL onto lysine-coated 96- or 6-well plates. Specifically, there were 1 × 10^5 cells/well in the 96-well plates (100 μL/well) and 1 × 10^5 cells/well in the 6-well plates (1 mL/well). Half of the medium was changed every three days.

**Assessment of Cell Purity**

Immunofluorescent staining of cultured cells was performed using antibody against MAP2 on 5th day after neuron isolation. Cells were rinsed with PBS, fixed in 4% paraformaldehyde in 0.01 mol/L PBS (pH 7.2) at room temperature for 25 min and washed three times with PBS for 5 min each. Cells were then incubated with 0.01% (v/v) Triton X-100 for 10 min and washed three times with PBS for 5 min each. Next, cells were blocked in goat serum at RT for 1 h, and primary monoclonal rat anti-MAP2 antibody diluted (1:200) with 1% (w/v) BSA dissolved in PBS (BSA/PBS) at 37 °C for 2 h. The cells were washed with three times with PBS, incubated with Alexa Fluor 594-conjugated goat anti-rat IgG (1:1000) for 1 h and incubated with 1 μg/mL Hoechst 33342 for 20 min, washed with PBS, and observed under an Olympus IX81 fluorescence microscope (OLYMPUS IX 81, Japan).

**OGD Model and AST-IV Treatment**

After 5–6 days in culture, the OGD/reoxygenation protocol was commenced; the culture medium was changed to low-glucose HBSS and the neurons were incubated in a hypoxic incubator with 1% O2, 5% CO2, and saturated humidity at 37 °C for 3 h. Following OGD, the neurons were reoxygenated in 95% air–5% CO2 and glucose-containing medium for 24 h prior to any further testing (Yanqiu et al., 2017). The treatment paradigm and timeline is illustrated in Fig. 1. For control cells (naive) that did not undergo OGD, the culture medium was replaced with regular glucose-containing culture medium and the cells were grown under normal oxygen conditions.
AST-IV was first diluted in low amounts of dimethyl sulfoxide (DMSO) ensuring that the final proportion of DMSO did not exceed 1/1000. Then low volumes of HBSS were repeatedly added and the AST-IV was slowly dissolved with warming and sonication. The final concentration of AST-IV was brought to 25, 12.5, and 6.25 μmol/L, which our preliminary dose-response tests found to be most efficient. The vehicle for AST-IV was 1/1000 DMSO in HBSS. AST-IV solution or vehicle was added to the neuronal cultures at the same time as the start of OGD and remained in the culture medium throughout the OGD and reoxygenation (Fig. 1).

Cell Viability and Cytotoxicity Assays

Following the OGD/reoxygenation of cortical neurons, we measured cell viability and cytotoxicity with and without AST-IV treatment. The viability of the primary neurons was evaluated by the CCK-8 assay. Briefly, 10 μL of CCK-8 was added to each well, and cells were further incubated for 1 h at 37 °C. Absorbance was then measured at 450 nm using a microplate reader (BioTek STNERGY™4, USA). Cytoxicity was determined by a detection assay that measured the release of lactate dehydrogenase (LDH) from cells to reflect levels of cytotoxicity. A 50-μL aliquot of medium from the samples was transferred to another new 96-well plate. Then equivalent dose of manufacturer-provided test reagent was added to each well for measurement of the release of LDH from the cells. Optical density was determined at 490 nm using a microplate reader.

Measurement of Mitochondrial Membrane Potential (ΔΨm) in Neurons

ΔΨm was estimated in the mitochondria of neurons using the JC-1 dye according to manufacturer’s instructions. JC-1 is a lipophilic, fluorescent cation that exists as green fluorescent monomers at low ΔΨm or as red fluorescent aggregates at high ΔΨm. Following OGD/reoxygenation, the neurons were incubated for 20 min at 37 °C in medium containing 10 μmol/L JC-1. Then, the neurons were rinsed twice in phosphate-buffered saline (PBS) and immediately analyzed by a microplate reader (BioTek STNERGY™4, USA) and visualized under a fluorescence microscope (OLYMPUS IX 81, Japan). Data was collected at 529 nm emission for green fluorescence and 590 nm for red fluorescence. The ratio of red/green fluorescence intensity represented the mitochondrial membrane potential.

ROS Quantification in Primary Neurons

To measure ROS levels, primary neurons were exposed to OGD by incubation in low-glucose HBSS under hypoxic conditions (1% O2, 5% CO2) for 3 h. Following OGD/reoxygenation, the neurons were incubated with 5 μmol/L Mitosox Red for 10 min and 1 μg/mL Hoechst 33342 was added for the last 5 min. Excess Mitosox Red and Hoechst 33342 were removed by washing the cells twice with PBS at room temperature. The fluorescence intensity was analyzed by a microplate reader (BioTek STNERGY™4, USA) and images were captured under a fluorescence microscope (OLYMPUS IX 81, Japan).

ATP-Release Assay in Neuronal Mitochondria

ATP release was detected using the ATPlite Luminescence ATP Detection Assay System (PerkinElmer EnSpire™, USA) according to the manufacturer’s protocol. Briefly, following OGD/reoxygenation and AST-IV treatment, neurons were washed with PBS. Then, they were incubated in 100 μL culture medium, to which 50 μL lysis buffer (from the ATPlite kit) was added for 5–10 min until neurons were completely fragmented. Finally, 50 μL substrate (from the ATPlite kit) was added for a 5 min incubation and the ATP content was measured on a multifunction microplate reader (Jarboe et al., 2012).

Western Blotting

The immunoblotting procedure was performed as previously described (Stephen et al., 2016). We used primary antibodies against cleaved caspase-3, caspase-3, p-PKA, PKA, p-CREB, CREB, and β-actin. HRP-conjugated goat anti-rabbit IgG was used as a secondary antibody. Briefly, Denatured proteins from neurons were separated by molecular weight using SDS-PAGE. The proteins in the gels were transferred to polyvinylidene fluoride membrane using a Trans-Blot Cell (Bio-Rad, USA). The membrane was blocked from nonspecific recognition of antibody with 5% skim milk or bovine serum albumin (for phosphorylated protein) dissolved in TBST. The proteins were detected using the indicated antibodies by means of an enhanced chemiluminescence system. Specially, the blot was stripped for phosphorylated proteins and blotted again for total proteins.

Statistical Analysis

All data were analyzed using SPSS 22.0 and presented as the means ± standard deviation (SD). Graphs were generated in GraphPad Prism 5. One-way analysis of variance (ANOVA) was performed to assess the statistical significance among multiple groups. Post-hoc pairwise comparisons were made by Tukey’s HSD if homogeneity of variance was met or
Games-Howell if the variance was unequal. Comparisons with $P < 0.05$ were considered to show a statistically significant difference.

**RESULTS**

**Verification of Cortical Neuronal Culture and AST-IV Doses**

In order to ensure that the proportion of astrocytes was relatively small in the culture system, we measured the immunofluorescence of the neuronal marker MAP2 and used Hoechst 33342 to stain all cell nuclei. As shown by the colocalization in Fig. 2A, we found that the majority of the cells in our culture preparation were neurons.

We screened the concentration of AST-IV from $3.125–50 \mu\text{mol/L}$ by CCK-8 assay to determine the general dose–response. As shown in the Fig. 2B, AST-IV produced little improvement in cell viability at $3.125 \mu\text{mol/L}$ and its response peaked at $25 \mu\text{mol/L}$ ($F (6, 28) = 43.0, P < 0.0001$; post-hoc Turkey, $P < 0.0001$ for OGD vs. AST-IV 25 and 50 $\mu\text{mol/L}$). For this reason, the dose range of 6.25 to 25 $\mu\text{mol/L}$ was eventually chosen for the experiments in our study.

**Effects of AST-IV on Primary Cortical Neuronal Activity, Morphology, and Apoptosis Induced by OGD**

OGD in neurons has been used as a rapid and sensitive in vitro model of ischemic stroke by many studies investigating potential neuroprotective agents (Wieloch et al., 2003; Nistico et al., 2009). To evaluate the protective effects of AST-IV against ischemic injury, we treated primary neurons with AST-IV and subjected them to OGD for 3 h. As shown in Fig. 3A, the 3-h OGD induced a significant decrease in cell viability (45.9% decline compared with control) determined by the CCK-8 assay. In contrast, AST-IV treatment (6.25, 12.5, and 25 $\mu\text{mol/L}$) induced a dose-dependent improvement in cell viability, with a 46.2% increase at 25 $\mu\text{mol/L}$ compared with that of the OGD group ($F (4, 20) = 51.5, P < 0.0001$; post-hoc Turkey, $P < 0.0001$ for OGD vs. AST-IV 25 $\mu\text{mol/L}$).

To confirm the neuroprotective effects of AST-IV, we conducted the LDH assay to assess OGD-induced cytotoxicity. AST-IV treatment significantly reversed LDH release in response to OGD ($F (4, 20) = 50.3, P < 0.0001$;...
post-hoc Turkey, $P < 0.0001$ for OGD vs. AST-IV 12.5, 25 μmol/L; $P = 0.005$ for OGD vs 6.25 μmol/L, Fig. 3B). Morphological examination of the cells revealed OGD-induced neuronal injuries characterized by the breakage of neuronal fibers and shrunken somas, which were partially reversed by AST-IV treatment (Fig. 3C). Furthermore, we performed immunoblots to examine the effects of AST-IV on OGD-induced neuronal apoptosis. Cleaved caspase-3 expression was increased in the OGD group but was reduced significantly in cells that received AST-IV treatment (F (4, 10) = 20.5, $P < 0.0001$; post-hoc Turkey, $P = 0.03$ for OGD vs. AST-IV 25 μmol/L, Fig. 3D). The change in Δψm is an indicator of the early stages of neuronal apoptosis and is described in the next section. Collectively, these results confirmed that AST-IV treatment improved neuronal viability and prevented OGD-induced apoptosis in primary neurons.

**Effects of AST-IV on OGD-Induced Mitochondrial Dysfunction**

To determine the potential mechanisms underlying the protective effects of AST-IV against OGD, we tested whether AST-IV could preserve mitochondria. As shown in Fig. 4A, we used JC-1 staining to detect the Δψm. The ratio of the aggregated
Fig. 4. The protective effects of AST-IV on mitochondria after OGD. (A) Δψm was examined using JC-1 dye and detected on a multifunctional microplate reader and fluorescence microscope. The polymeric form of JC-1 (red) appeared in mitochondria with high membrane potential, whereas its monomeric form (green) appeared in mitochondria with low membrane potential. The ratios of polymeric and monomeric forms of JC-1 were quantified in each group and presented as a fold change from the OGD group. n = 4. (B) ROS production was measured in neurons with MitoSOX Red staining, with Hoechst 33342 to label all cell nuclei. Representative images are shown (original magnification, 20×) with a higher magnification (100×) image in each inset. The quantified levels of ROS are expressed as the means ± SD. n = 5. (C) Neuronal ATP production was measured by an ATPlite assay and the values are expressed as the fold difference from the ATP level of the OGD group. n = 5. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the OGD group.
Effects of AST-IV on the PKA/CREB Signaling Pathway

It has been reported that PKA/CREB was involved in preserving mitochondrial function and regulating ROS and ATP levels in mitochondria (Signorile et al., 2014; Xie et al., 2017). Furthermore, the expression of PKA/CREB was down-regulated during cerebral ischemia (Niu et al., 2014). Since this study (see the section above) and other studies (He et al., 2012) have reported that AST-IV can protect mitochondrial function, we hypothesized that AST-IV regulates the PKA/CREB pathway under conditions of OGD. The phosphorylation of CREB and PKA following a 3-h OGD was examined by western blotting using antibodies that specifically recognize the activated (phosphorylated) forms of PKA and CREB. Fig. 5A shows a significant decline of phosphorylated CREB and PKA in the OGD group (declines of 68.3% and 79.0%, respectively), suggesting that PKA/CREB signaling was down-regulated by the ischemic injury. In contrast, the AST-IV treatment markedly activated PKA and CREB in a dose-dependent manner (for PKA, F (4, 10) = 65.4, P < 0.0001; post-hoc Turkey, P = 0.031 for OGD vs. AST-IV 12.5 μmol/L; for CREB, F (4, 10) = 35.3, P < 0.0001; post-hoc Turkey, P = 0.010 for OGD vs. AST-IV 25 μmol/L, Fig. 5A). Since CREB can be activated by several different protein kinases, we used H-89, a PKA inhibitor, to confirm whether AST-IV-induced CREB phosphorylation was elicited specifically by PKA. In the presence of 10 μmol/L H-89 under conditions of OGD and reoxygenation (Katarzyna et al., 2017; Zhong et al., 2018), the increased levels of p-CREB induced by AST-IV were significantly blocked (F (5, 12) = 42.9, P < 0.0001; post-hoc Turkey, P = 0.018 and a 38.2% declined for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L, Fig. 5B). Together, these data indicated that activation of PKA was a prerequisite for the AST-IV-mediated enrichment of p-CREB, which is an important nuclear and mitochondrial protein that modulates neuron survival, memory, and cognitive function.

Role of PKA in AST-IV-Mediated Mitochondrial Protection

We have shown above that AST-IV alleviated mitochondrial injury caused by OGD, and that OGD-induced decline in PKA and CREB phosphorylation was reversed by AST-IV treatment. Thus, we hypothesized that the beneficial effects of AST-IV (regulating ROS and ATP, regulating mitochondrial potential, and improving neuronal viability) were induced by de-repression of the PKA/CREB signaling pathway. In support of this hypothesis, the PKA inhibitor, H-89, partially suppressed the effects of AST-IV on ATP production (F (4, 20) = 20.3, P < 0.0001; post-hoc Turkey, P = 0.007 for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L, Fig. 6B) and mitochondrial potential (F (4, 20) = 45.7, P < 0.0001; post-hoc Games-Howell, P = 0.003 for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L, Fig. 6C), suggesting that PKA/CREB signaling directly modulated mitochondrial activity. Furthermore, the effects of AST-IV on neuronal viability and cytotoxicity were also partially blocked by H-89 for CCK-8 assay, F (4, 20) = 54.2, P < 0.0001; for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L; for LDH assay, F (4, 20) = 73.9, P < 0.0001; post-hoc Turkey, P = 0.009 for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L (Fig. 6D and E), indicating that AST-IV targeted the PKA/CREB pathway to reverse OGD-induced mitochondrial dysfunction and neuronal death. Conversely, H-89 exposure did not block the effect of AST-IV on ROS generation (F (4, 20) = 26.3, P < 0.0001; post-hoc Turkey, P = 0.763 for AST-IV 25 μmol/L + H-89 vs. AST-IV 25 μmol/L, Fig. 6A).

DISCUSSION

Ischemic stroke leads to neuronal dysfunction and cognitive impairment (Mijajlović et al., 2017). The current clinical treatments for ischemic stroke are mostly limited to thrombolytics, such as recombinant tissue plasminogen activator, that have a very restricted window for success following the injury (Zerna et al., 2016). Mitochondrial impairment was found to be one of the key pathological events that occur in the early stages of ischemic injury (Aggarwal et al., 2010; Kahl et al., 2018). Previous studies have elucidated the critical role of mitochondria in the cell; this organelle fulfills numerous cellular functions including ATP production, buffering of calcium ions (Ca²⁺), ROS production and sequestration, apoptosis, and neurotransmitter synthesis and inactivation (Rowley et al., 2012; Tait and Green, 2013; Brini et al., 2014; Žorov et al., 2014). However, mitochondria were shown to be vulnerable to ischemic injury due to the accompanying deficiency in oxygen and glucose (Novgorodov et al., 2016), and mitochondria-related potential therapies for stroke are gaining traction in the field. Decreased mitochondrial membrane potential is an indication that the mitochondrial membrane has been permeabilized, which is an early sign of OGD-induced neuronal apoptosis (Parra et al.,
Accumulating evidence suggests a close relationship between excessive ROS production and neuronal death in various neurological disorders, including amyotrophic lateral sclerosis, Alzheimer’s disease (AD), Parkinson’s disease (PD), ischemic stroke, and traumatic brain injury (Cadenas and Davies, 2000; Valko et al., 2007). Mitochondrial dysfunction has been shown to cause the generation of the highly reactive species peroxynitrite (ONOO−), which led to excessive ROS production and further inhibited the function of mitochondrial complexes (Dugan et al., 2009; Aggarwal et al., 2010). Moreover, production of ROS in this way can damage virtually every biomolecule found in cells, promote opening of mitochondrial PTPs, and activate inflammatory and thrombogenic cascades to exacerbate cell injury (Theodore et al., 2016). Many natural agents, such as AST-IV and Ginkgo biloba extract, show promising antioxidant activity (Z. Wang et al., 2017; H.L. Wang et al., 2017; Zhou...
et al., 2017). Furthermore, mitochondrial superoxide dismutase, an enzyme that neutralizes ROS, as well as antioxidants such as azulenyl nitrones, have shown promising results in preclinical studies of ischemic stroke (Ginsberg et al., 2003; Tsubokawa et al., 2007). Some reports have considered that antioxidants may modulate mitochondrial PKA and increase CREB binding to D-loop DNA of the mitochondrial genome in neurons (Hoon et al., 2005; Zhou et al., 2017). Methylene blue, which enhances mitochondrial ATP production and propogates the electron transport chain, has been shown to reduce infarct size and improve cerebral blood flow (Lora et al., 2013).

AST-IV is one of the most important bioactive compounds of the herb Radix Astragali that is widely used in Chinese traditional medicine for the treatment of cerebrovascular diseases (Hu et al., 2012; Ren et al., 2013). The pharmacological

**Fig. 6.** H-89 partially blocked the effects of AST-IV on OGD-exposed mitochondria and neurons. (A, B) OGD-induced ROS and ATP levels were determined with or without H-89 following AST-IV treatment. n = 5. (C, D, E) Effects of AST-IV on ΔΨm (JC-1 staining), cell viability (CCK-8), and cytotoxicity (LDH-release assay) with or without H-89. n = 4. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the OGD group. #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with the OGD + AST-IV group.
effects of AST-IV include cardiac protective function (Chen et al., 2011), anti-asthma (Du et al., 2008), and anti-stroke properties (Li et al., 2013). In this study, we demonstrated that AST-IV improved mitochondrial activity in cortical neurons subjected to OGD; it regulated the generation of ROS and ATP, and inhibited OGD-induced neuronal apoptosis. These findings revealed a mitochondria-mediated mechanism underlying the neuroprotective effects of AST-IV and provided further evidence to support the use of Radix Astragali in the treatment of ischemic stroke.

The activity of mitochondria has been shown to be regulated by PKA/CREB signaling (Lee et al., 2005; Ryu et al., 2005). Numerous studies have demonstrated the essential role of CREB in memory and cognitive function (Tully, 1997; Saura and Cardinaux, 2017). CREB was reported to regulate a variety of intracellular signaling events, including cellular growth, proliferation, synaptic efficacy, and long-lasting changes in synaptic plasticity (Meyer et al., 1988; Babikian et al., 1990; Dugan et al., 2009). PKA is an upstream component of the signaling pathway with CREB that can modulate CREB activity by phosphorylating it on Serine 133 (Naqvi et al., 2014). CREB can not only trigger gene expression in the nucleus but was also reported to exist in the mitochondrial matrix of neurons to ensure efficient signal propagation between the neuronal membrane and mitochondria (Lee et al., 2005; Ryu et al., 2005). The PKA/CREB signaling pathway was shown to modulate ROS and ATP generation to preserve mitochondria (Ryu et al., 2005).

The links between AST-IV, ischemic stroke, mitochondrial dysfunction, and PKA/CREB signaling prompted our hypothesis that AST-IV protects OGD-exposed neurons from injury and death by preserving mitochondrial function via the regulation of PKA/CREB pathway. The results demonstrated that the protective effects of AST-IV were partially blocked by PKA inhibitor H-89, indicating that AST-IV protected mitochondria and enhanced neuronal viability by activating the PKA/CREB pathway. Previous studies have shown that AST-IV attenuated cognitive impairment caused by stroke (Kim et al., 2015; Li et al., 2017); however, the associated mechanism was never elucidated. In this study, we found that the OGD-induced decrease in CREB was reversed by AST-IV treatment. Our observations of AST-IV’s regulation of CREB and its requirement of PKA elucidated one of the mechanisms underlying its protection against OGD-induced ischemic injury.

Based on the results of this study, we believe that PKA/CREB was the primary pathway targeted by AST-IV; however we also observed that H-89 exposure did not block the positive effect of AST-IV on ROS production, which indicated that PKA was not the only kinase by which AST-IV acted. In fact, it has been reported that regulatory kinases of CREB also included MAPK, AKT, and CaMKII (C. Unsöld et al., 1998). In addition, AST-IV treatment of neurons subjected to H2O2 showed an increase in phosphorylated p38 MAPK (Liu et al., 2017). Therefore, PKA-independent signaling pathways should also be investigated in the future to comprehensively understand the mechanisms underlying AST-IV’s beneficial effects in ischemic injury.

The activity of PKA is dependent on the cellular level of cAMP (Gorshkov et al., 2017). We speculate that AST-IV triggers neuroprotection via the PKA/CREB pathway by binding to G-protein coupled receptors that regulate cAMP activity. Several reports in the literature support this idea: (1) The specific receptors that interact with AST-IV are associated with protease-activated receptor-2 (PAR2) (Z. Wang et al., 2017; H.L. Wang et al., 2017), G protein-coupled receptor kinase-2 (GPRK2)-CXCR2 (Huang et al., 2016), CXCR4 (Xie et al., 2013), and CC chemokine receptor 3 (CCR3) (Gu et al., 2012), all of which are G-protein coupled receptors. (2) It was also reported that the PKA/CREB pathway lay downstream of a G-protein coupled receptor (Bauman et al., 2006). Therefore, we believe that following an ischemic injury, AST-IV may interact with the G-protein coupled receptors, promote the membrane-associated adenylyl cyclase to generate cAMP, activate the PKA/CREB pathway, regulate the generation of ROS and ATP, and finally inhibit neuronal apoptosis and increase neuronal survival.

While we successfully revealed a mechanism of AST-IV with an in vitro model of ischemic injury, we did not use animal models to verify this PKA/CREB/mitochondria-mediated protective pathway in ischemia. However, other animal studies of AST-IV in ischemia indirectly corroborated our findings. One study in rats subjected to ischemia/reoxygenation injury revealed that AST-IV protected brain tissue by inhibiting peripheral benzodiazepine receptors (PBRs) (Cao et al., 2015), which are found in the mitochondrial outer membrane and are involved in oxidative processes and mitochondrial function (Casellas et al., 2002). Another study reported that AST-IV accomplished its neuroprotective effects in ischemic mice by blocking the activation of the NLRP3 inflammasome (Yu and Lee, 2016), a downstream consequence of mitochondrial dysfunction and excess ROS production (Yu and Lee, 2016).

In summary, we have shown that AST-IV protected against OGD-induced apoptosis in primary neurons by regulating the PKA/CREB pathway and preserving mitochondrial function. As the OGD-induced down-regulation of CREB plays a critical role in cognitive impairment associated with ischemic injury, our study strongly suggests that AST-IV could be effective in treating cognitive impairments associated with ischemic stroke. Our findings provide a better understanding of the molecular mechanisms underlying the neuroprotective effects of AST-IV and could offer new insight into potential therapeutic targets to treat ischemic injury.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support from the National Natural Sciences Foundation of China (81703747), National Key Basic Research Program of China (973 Program) (2015CB554400), and the China Postdoctoral Science Foundation (2017 M621041).

CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest regarding the publication of this article.
REFERENCES
