Regular Article

Acteoside Attenuates Oxidative Stress and Neuronal Apoptosis in Rats with Focal Cerebral Ischemia–Reperfusion Injury

Dongjian Xia,^{a,b} Zhuang Zhang,^c and Yuanli Zhao^{*a}^aDepartment of Neurosurgery, Beijing Tian Tan Hospital, Capital Medical University; Beijing 100050, P. R. China:^bDepartment of Neurosurgery, the First Affiliated Hospital, Jinzhou Medical University; Jinzhou 121001, P. R.China; and ^cDepartment of Neurology, the First Affiliated Hospital, Jinzhou Medical University; Jinzhou 121001, P. R. China.

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Acteoside (ACT) has been shown to exert antioxidant and neuroprotective effects in neurodegenerative diseases. However, the effect of ACT on cerebral ischemia–reperfusion (I/R) injury is not yet clear. In this study, we found that ACT administration reduced infarct volume and brain edema, and improved neurological deficits, as indicated by the decreased modified neurological severity score. Administration of ACT strikingly reduced oxidative stress, accompanied by decreased levels of reactive oxygen species and malondialdehyde and increased levels of superoxide dismutase and catalase in a rat model of middle cerebral artery occlusion/reperfusion (MCAO/R). Furthermore, ACT administration reduced the number of terminal deoxynucleotidyl transferase uridine 5'-triphosphate (UTP) nick-end labeling-positive cells in the cerebral cortex of ischemic side of MCAO/R rats, accompanied by downregulation of B cell lymphoma 2 (Bcl-2) associated X protein and cleaved caspase-3 proteins and upregulation of Bcl-2 protein. Additionally, ACT treatment inhibited the protein kinase R/eukaryotic initiation factor-2 α stress pathway in the brains of MCAO/R rats. Our results demonstrated that ACT attenuates oxidative stress and neuronal apoptosis in MCAO/R rats, suggesting that ACT may serve as a novel therapeutic candidate for the treatment of I/R brain injury.

Key words acteoside; middle cerebral artery occlusion–reperfusion; oxidative stress; apoptosis

Stroke is one of the most common neurological disorders, and is the second largest cause of death and disability worldwide.¹⁾ Based on its pathogenesis, stroke can be classified into 2 categories: ischemic stroke and hemorrhagic stroke. Ischemic stroke results from disturbances of the cerebral circulation, while hemorrhagic stroke occurs as a result of alterations to the blood vessels in the brain, which ultimately cause intraparenchymal or subarachnoid hemorrhage.^{2,3)} Of all adult strokes, approximately 80% are ischemic.⁴⁾ Currently, the best treatment for ischemic stroke is thrombolytic therapy.⁵⁾ However, restoration of blood circulation to the ischemic brain is not always effective. Conversely, it may cause an ischemia–reperfusion (I/R) injury.⁶⁾ Thus, developing strategies to ease the injury caused by cerebral I/R is the focal point of research at present.

Acteoside (ACT) or verbascoside is a caffeoyl phenylethanoid glycoside, which exists in multifarious plants, such as *Cistanche* spp., *Pithecoctenium* spp., and *Plantago* spp. ACT has been shown to exhibit neuroprotective activities in neurodegenerative diseases. For example, ACT protected SH-SY5Y cells against amyloid-beta peptides (A β)_{25–35}-induced cell injury through attenuating reactive oxygen species (ROS) production, reducing apoptosis, and improving mitochondrial membrane potential.⁷⁾ Furthermore, ACT markedly repressed the aggregation of a 42-mer amyloid β protein in a concentration-dependent manner.⁸⁾ In addition, ACT attenuated D-galactose-induced cerebral damage by inhibiting the increased expression of glial fibrillary acidic protein and neurotrophin-3.⁹⁾ Besides, ACT mitigated amyloid β peptide-induced cognitive dysfunction, neurotoxicity, and neurochemical disturbances *via* reducing amyloid protein deposition.¹⁰⁾ However, whether ACT exerts its neuroprotective effect in cerebral I/R injury

remains elusive.

Protein kinase R (PKR), also known as double-stranded RNA (dsRNA)-activated protein kinase, has a C-terminal kinase domain and an N-terminal dsRNA-binding domain, which gives it pro-apoptotic functions.¹¹⁾ PKR plays an important role in cellular responses to a variety of stimuli, such as oxidative stress, pathogens, and endoplasmic reticulum stress.¹²⁾ Eukaryotic initiation factor-2 α (eIF2 α), the downstream signaling molecule of PKR, plays a key role in initiation of protein translation, thereby modulating cellular signaling.¹³⁾ Under normal conditions, PKR is expressed constitutively but is not functional. Under stress conditions, activated PKR directly phosphorylates eIF2 α at Ser51, which leads to inhibition of mRNA translation and ultimately results in apoptosis.¹⁴⁾ Recently, the PKR/eIF2 α pathway has been shown to be closely associated with the occurrence and development of various neurological and neuropsychiatric disorders. However, its role in cerebral I/R injury is still obscure.

In the present study, we found that ACT administration reduced infarct volume and brain water content and improved neurological deficits in middle cerebral artery occlusion/reperfusion (MCAO/R) rats. Moreover, our study concluded that ACT attenuates oxidative stress and neuronal apoptosis. These findings suggest that ACT may serve as a potential therapeutic agent for the treatment of cerebral I/R injury.

MATERIALS AND METHODS

Animals All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Beijing Tian Tan Hospital. Thirty male Wistar rats weighing 300–350 g were purchased from Vital River Lab Animal

*To whom correspondence should be addressed. e-mail: zhaoylth@yeah.net

Technology Co., Ltd. (Beijing, China), and kept under standard conditions of temperature ($22 \pm 2^\circ\text{C}$), relative humidity ($50 \pm 10\%$), and light (12/12h light/dark cycle). Rats had free access to a standard rodent diet *ad libitum*. Rats were distributed randomly as follows: Sham group: MCAO/R surgery was performed but no monofilament was advanced into the origin of the middle cerebral artery; MCAO/R group: MCAO/R surgery was performed; MCAO/R+ACT (10mg/kg/d) group: MCAO/R surgery was performed and ACT (10mg/kg/d; Catalog No. CFN97048; ChemFaces, Wuhan, China) was administered orally for 3 consecutive days; MCAO/R+ACT (20mg/kg/d) group: MCAO/R surgery was performed and ACT (20mg/kg/d) was administered orally for 3 consecutive days; MCAO/R+ACT (40mg/kg/d) group: MCAO/R surgery was performed and ACT (40mg/kg/d) was administered orally for 3 consecutive days.

MCAO Surgery Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350mg/kg). Rectal and temporalis muscle temperatures were monitored and maintained at $37 \pm 0.5^\circ\text{C}$. The rat was placed on its back and the right carotid artery was exposed through a neck incision. A 4-0 monofilament nylon thread with an expanded tip was then introduced into the internal carotid artery to block the origin of the middle cerebral artery. After 2h of occlusion, the monofilament was gently withdrawn to commence reperfusion.

Brain Edema Measurement At 24h after the last ACT treatment, brain edema was assessed using the dry/wet

method. The brain of each rat was moved and placed in a pre-weighed dish to measure its wet weight (WW). Subsequently, the dish and the rat brains were desiccated in an oven at 110°C for 24h to determine the dry weight (DW). Brain water content (%) was determined using the following equation: $[(\text{WW} - \text{DW}) / \text{WW}] \times 100$.

Measurement of Infarcted Volume To measure the infarct volume, rats were sacrificed and their brains were removed. Each brain was cut into 2mm thick slices, and stained with 2,3,5-triphenyltetrazolium chloride (TTC; Beyotime, Shanghai, China) at 37°C for 20min. Subsequently, the slices were fixed in 10% formalin solution. The infarct volume was measured using ImageJ software (National Institutes of Health, NY, U.S.A.) and calculated as the sum of areas of 6 slices from each rat. Results were presented as a percentage of the infarct area.

Modified Neurological Severity Score (mNSS) At 24h after the last ACT treatment, mNSS test was performed to assess neurological deficits. This test evaluated 4 aspects of neurological function, including motor, sensory, reflex, and balance functions. Neurological deficit was assessed using a 0–18 point rating scale (normal score: 0; maximal deficit score: 18). High scores indicated severe neurological deficit.

Enzyme-Linked Immunosorbent Assay (ELISA) The rat brains were homogenized in phosphate buffered saline (PBS) and centrifuged at $5000 \times g$ for 5min at 4°C . The supernatant was used to determine the total ROS content using Rat

Fig. 1. Effect of ACT on Infarct Volume and Neurological Deficits in MCAO/R Rats

(A) The structure of ACT has been illustrated. After MCAO/R treatment, various doses of ACT (10, 20, and 40mg/kg/d) were orally administered to MCAO/R rats for 3 consecutive days. Neurological deficits were assessed using TTC staining, standard wet-dry method, and mNSS test. (B and C) ACT treatment markedly reduced infarct volume and brain edema. (D) ACT administration improved neurological deficits, as indicated by reduction of mNSS scores. * $p < 0.05$, ** $p < 0.01$.

ROS ELISA kit (Wuhan EIAab Science, Wuhan, China), in accordance with the manufacturer's instructions.

Measurement of Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Catalase (CAT) Levels The levels of MDA, SOD, and CAT in the rat brains were measured using commercial assay kits (Solarbio, Beijing, China), in accordance with the manufacturer's instructions.

Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate (dUTP) Nick-End Labeling (TUNEL) Assay Apoptosis was detected using TUNEL assay. Briefly, the brain tissues sections were fixed in 4% paraformaldehyde (Solarbio) for 15 min, and then treated with 0.1% (v/v) Triton X-100 (Solarbio) for 3 min. Subsequently, the sections were incubated with TUNEL reaction mixture for 1 h at 37°C in the dark. Following this, sections were counterstained with 6-diamidino-2-phenylindole (DAPI; Solarbio) for 5 min at 25°C in the dark. The number of TUNEL-positive cells were counted under a fluorescent microscope (Olympus, Tokyo, Japan). Results were expressed as percentage of TUNEL-positive cells.

Western Blotting The cerebral cortex of ischemic side was removed, grinded, and sonicated in lysis buffer at 4°C. Protein concentration of the lysate was determined using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). Protein extracts (50 µg) were resolved on 8% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk for

2 h at room temperature, the membranes were probed with primary antibodies against B cell lymphoma 2 (Bcl-2) associated protein (Bax) (Abcam, Cambridge, MA, U.S.A.), Bcl-2 (Abcam), cleaved caspase-3 (Abcam), pPKR_{Thr446} (Abcam), PKR (Abcam), pEIF2 α _{Ser51} (Cell Signaling Technology, Beverly, MA, U.S.A.), eIF2 α (Cell Signaling Technology), and β -actin (Sigma-Aldrich, Louis, MO, U.S.A.) overnight at 4°C. After rinsing in PBS, the membranes were immunoblotted with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h at room temperature. The membranes were developed using ECL reagents (Pierce), and protein bands were quantified using the ImageJ software.

Statistical Analysis All data analyses were performed using SPSS 20.0 software (SPSS, Chicago, IL, U.S.A.). Values were presented as mean $\pm$ standard deviation (S.D.) representative of three independent experiments. Differences between groups were analyzed using one-way ANOVA followed by Tukey's *post hoc* test, and were considered statistically significant when $p < 0.05$.

RESULTS

The Neuroprotective Effect of ACT against Cerebral I/R Injury in Rats The molecular structure of ACT is shown in Fig. 1A. To assess the neuroprotective effect of

Fig. 2. ACT Attenuates Oxidative Stress in MCAO/R Rats

After MCAO/R treatment, ACT (40 mg/kg/d) was orally administered to MCAO/R rats for 3 consecutive days. The levels of ROS (A), MDA (B), SOD (C), and CAT (D) in the cerebral cortex of ischemic side were then measured. ACT administration reduced the levels of ROS and MDA, and increased the levels of SOD and CAT in MCAO/R rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. ACT Prevents Neuronal Apoptosis in MCAO/R Rats

(A) Quantification of TUNEL-positive cells in the cerebral cortex of ischemic side of MCAO/R rats. ACT administration reduced the number of TUNEL-positive cells compared with the MCAO/R group. Representative images of Western blot analysis and quantification of Bax (B), Bcl-2 (C), and cleaved caspase-3 (D) expression in the cerebral cortex of ischemic side of MCAO/R rats. ACT administration reduced the protein levels of Bax and cleaved caspase-3, but increased the Bcl-2 protein level in the cerebral cortex of ischemic side of MCAO/R rats. ** $p < 0.01$, *** $p < 0.001$.

ACT, various doses of ACT (10, 20, and 40 mg/kg/d) were orally administered to MCAO/R rats for 3 consecutive days. The infarcted area of the brain was assessed by TTC staining. Results showed that ACT administration (10, 20, and 40 mg/kg/d) resulted in reduced infarcted volume and brain edema compared to that in MCAO/R rats, especially at a dose of 40 mg/kg/d (Figs. 1B, 1C). Thus, a dose of 40 mg/kg/d of ACT was selected for all following experiments. Correspondingly, ACT administration (40 mg/kg/d) resulted in an obvious decrease in mNSS compared with that in MCAO/R rats (Fig. 1D). Therefore, our study revealed that ACT reduced infarcted volume and brain edema and improved neurological deficits in MCAO/R rats.

ACT Attenuates Oxidative Stress in I/R Rats To investigate the effect of ACT on oxidative stress in MCAO/R rats, ROS, MDA, SOD, and CAT levels were measured. ROS and MDA levels were remarkably increased in the MCAO/R group compared with those in the sham group, but decreased in the ACT group as compared with the MCAO/R group (Figs. 2A,

2B). Conversely, the levels of SOD and CAT were strikingly reduced in the MCAO/R group compared with those in the sham group, but increased in the ACT group as compared with the MCAO/R group (Figs. 2C, 2D). Together, these data indicated that ACT attenuated oxidative stress in MCAO/R rats.

ACT Prevents Neuronal Apoptosis in MCAO/R Rats

To gain insights into the anti-apoptotic effect of ACT, we explored the effect of ACT on neuronal apoptosis in MCAO/R rats using TUNEL assay. As expected, an obvious increase in the number of TUNEL-positive cells was observed in the MCAO/R group compared with that in the sham group, but this effect was blocked by ACT administration (Fig. 3A). To further elucidate the mechanism by which ACT elicits its anti-apoptotic effect, the expression levels of apoptosis-related proteins, including Bax, Bcl-2, and cleaved caspase-3 were determined by Western blotting. Results showed that the protein levels of Bax and cleaved caspase-3 in the MCAO/R group were strikingly higher compared to those in the sham group.

Fig. 4. ACT Inhibits the PKR/eIF2 α Stress Pathway

(A) Representative images of Western blot analysis and quantification of pPKR_{Thr446} and PKR expression in the cerebral cortex of ischemic side of MCAO/R rats. (B) Representative images of Western blot analysis and quantification of pEIF2 α _{Ser51} and eIF2 α expression in the cerebral cortex of ischemic side of MCAO/R rats. ** $p < 0.01$, *** $p < 0.001$.

The protein levels of Bax and cleaved caspase-3 in the ACT group were lower compared to those in the MCAO/R group (Figs. 3B, 3D). In contrast, the protein level of Bcl-2 was decreased in the MCAO/R group compared with that in the sham group, but increased in the ACT group compared with that in the MCAO/R group (Fig. 3C). These results revealed that ACT protects neurons against MCAO/R-induced apoptosis.

ACT Inhibits the PKR/eIF2 α Stress Pathway To verify whether the neuroprotective effect of ACT was mediated by the PKR/eIF2 α stress pathway, the expression of pPKR_{Thr446}, PKR, pEIF2 α _{Ser51}, and eIF2 α in the brain of MCAO/R rats was examined by Western blotting. As shown in Figs. 4A and 4B, MCAO/R treatment induced a striking increase in the protein levels of pPKR_{Thr446} and pEIF2 α _{Ser51}. However, administration of ACT suppressed the MCAO/R-induced phosphorylation of PKR and eIF2 α . Collectively, our findings suggested that ACT inhibited the PKR/eIF2 α stress pathway.

DISCUSSION

Oxidative stress has been implicated in brain damage and neuronal apoptosis during cerebral I/R.¹⁵ Excessive production of ROS in neurons plays a crucial role in the development of cerebral I/R injury. Due to weak antioxidant enzyme activity, the brain is vulnerable to oxidative damage elicited by cerebral I/R injury, which ultimately leads to lipid peroxidation and ultimately neuronal apoptosis.¹⁶ Oxidative stress causes disturbances in cerebral microcirculation and increases blood-brain barrier permeability, thereby resulting in brain edema.¹⁷ Besides, in a previous study, oxidative stress led to disturbances in excitatory amino acid metabolism, resulting in excitotoxic injury.¹⁸ Moreover, ROS can activate inflammatory cells through several mechanisms, which further aggravate brain injury.¹⁹ Therefore, exploring drugs that attenuate oxidative stress-induced injury is very important. In this study, we found that ACT treatment reduced infarct volume and

brain water content and decreased the mNSS in MCAO/R rats. Moreover, ACT administration mitigated oxidative stress, as indicated by decreased levels of ROS and MDA and increased levels of SOD and CAT.

Apoptosis is an autonomic ordered programmed cell death in order to maintain homeostasis, which is controlled by polygenes.^{20,21} Several lines of evidence have shown that apoptosis plays an important role in the pathogenesis of various human diseases, including cerebral I/R injury. During cerebral ischemia, both cyclooxygenase-2 and inducible nitric oxide synthase are highly expressed, and induce activated microglial cells to generate ROS.²² Overproduction of ROS leads to increased mitochondrial membrane permeability, resulting in translocation of Bax from the cytosol to the mitochondria and release of cytochrome *c*.²³ Once released into the cytosol, cytochrome *c* binds to apoptotic protease-activating factor 1, which together with procaspase-9 forms a complex termed the apoptosome. This complex activates procaspase-9 and -3 and ultimately results in neuronal apoptosis.²⁴ Therefore, targeting and preventing neuronal apoptosis in the brain appears to be a promising therapeutic strategy for the treatment of cerebral I/R injury. In the present study, ACT administration suppressed MCAO/R-induced neuronal apoptosis, as indicated by decreased Bax and cleaved caspase-3 levels and increased Bcl-2 levels, which was in accordance with a reduction in the number of TUNEL-positive cells.

There is growing evidence to suggest that the PKR/eIF2 α stress pathway is involved in the pathology of neurodegenerative disorders. For example, Mouton-Liger *et al.* demonstrated that the levels of pPKR and pEIF2 α were increased in SH-SY5Y cells exposed to H₂O₂.²⁵ Chang *et al.* showed that activation of the PKR/eIF2 α pathway was associated with neuronal apoptosis in Alzheimer's disease.²⁶ Zhang *et al.* suggested that gatrodin alleviated memory deficits *in vivo* and inhibited beta-amyloid converting enzyme 1 expression *via* inhibition of the PKR/eIF2 α pathway.²⁷ Ma *et al.* reported

that chronic copper exposure might induce spatial memory impairment, selective loss of hippocampal synaptic proteins, and apoptosis through activation of the PKR/eIF2 α pathway.²⁸⁾ Vaughn *et al.* demonstrated that inhibition of PKR delayed phosphorylation of eIF2 α and protected EV and K11 cells from tunicamycin-induced apoptosis in neuronal cells.²⁹⁾ It has been reported that the apoptotic caspase-3, caspase-7, and caspase-8 could cleave PKR, but the inflammatory caspase-1 and caspase-11 could not. During the apoptosis process, PKR activation and eIF2- α phosphorylation occur in a caspase-dependent manner.³⁰⁾ Caspase-3 can cleave the alpha subunit of eIF-2, thus modulating the efficiency of protein synthesis.³¹⁾ In our study, a progressive enhancement of pPKR_{Thr446}/PKR and pEIF2 α _{Ser51}/eIF2 α ratio was observed in the cerebral cortex of ischemic side of MCAO/R rats. Importantly, ACT administration inhibited the phosphorylation of PKR and eIF2 α at Thr446 and Ser51, respectively.

In conclusion, our study demonstrated that ACT administration reduced infarct volume and brain water content and improved neurological deficits. We also showed that ACT treatment attenuated the brain damage induced by cerebral I/R through mitigating oxidative stress and neuronal apoptosis. Our findings indicate that ACT may serve as a potential therapeutic agent for the treatment of cerebral I/R injury.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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