RESEARCH ARTICLE

ESE1 expression correlates with neuronal apoptosis in the hippocampus after cerebral ischemia/reperfusion injury

Hai-Long Yu[1, 2, 3, 4, #], **Liang-Zhu Wang**[5, #], **Ling-Ling Zhang**[1, 2], **Bei-Lei Chen**[1, 2], **Hui-Juan Zhang**[1, 2], **Yu-Ping Li**[1, 2], **Guo-Dong Xiao**[6, *], **Ying-Zhu Chen**[1, 2, *]

1 Clinical Medical College of Yangzhou University, Yangzhou, Jiangsu Province, China
2 Department of Neurology, Northern Jiangsu People's Hospital, Yangzhou, Jiangsu Province, China
3 Institute of Neuroscience, Northern Jiangsu People's Hospital, Yangzhou, Jiangsu Province, China
4 Drum Tower Hospital, Medical School of Nanjing University, Nanjing, Jiangsu Province, China
5 Dalian Medical University, Dalian, Liaoning Province, China
6 Department of Neurology, Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, China

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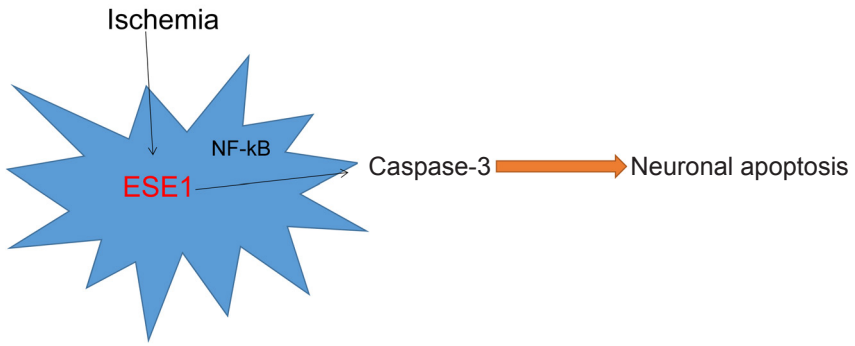
Graphical Abstract

Epithelial-specific ETS-1 (ESE1) associates with neuronal apoptosis after cerebral ischemia/reperfusion *via* activating nuclear factor-κB (NF-κB) signaling pathway



Correspondence to:
Guo-Dong Xiao, PhD, yarrowshaw@hotmail.com;
Ying-Zhu Chen, PhD, yzchendr@163.com.

 # These authors contributed equally to this work.

orcid:
0000-0001-9448-3127 (Guo-Dong Xiao)
0000-0002-2110-236X (Ying-Zhu Chen)

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Abstract

Epithelial-specific ETS-1 (ESE1), a member of the ETS transcription factor family, is widely expressed in multiple tissues and performs various functions in inflammation. During neuroinflammation, ESE1 promotes neuronal apoptosis; however, the expression and biological functions of ESE1 remain unclear after cerebral ischemia/reperfusion. We performed *in vivo* and *in vitro* experiments to explore the role of ESE1 in cerebral ischemic injury. A modified four vessel occlusion method was used in adult Sprague-Dawley rats. At 6, 12, 24, 48, and 72 hours after model induction, the hippocampus was collected for analysis. Western blot assays and immunohistochemistry showed that the expression of ESE1, phosphorylated p65 and active caspase-3 was significantly up-regulated after ischemia. Double immunofluorescence staining indicated that ESE1 and NeuN were mostly co-located in the hippocampus after ischemia. Furthermore, ESE1 was also co-expressed with active caspase-3. PC12 cells were stimulated with cobalt chloride ($CoCl_2$) to establish a chemical hypoxia model. After ESE1 knockdown by siRNA for 6 hours, cell viability was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assays. The levels of ESE1, phosphorylated p65 and active caspase-3 were also remarkably increased in PC12 cells after $CoCl_2$ stimulation. After ESE1 knockdown, PC12 cell viability was increased after hypoxia. siRNA knockdown of ESE1 decreased the level of p-p65 and active caspase-3 after $CoCl_2$ stimulation. These data reveal that ESE1 levels are elevated in the hippocampus after cerebral ischemia/reperfusion injury. This may play a role in neuronal apoptosis *via* activation of the nuclear factor-κB pathway.

Key Words: *stroke; epithelial specific ETS-1; cerebral ischemia/reperfusion; nuclear factor-κB; inflammation; caspase-3; neuroprotective effects; neural regeneration; cobalt chloride ($CoCl_2$); siRNA transfection*

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Introduction

Stroke has become the second most common cause of mortality in adults worldwide (Lozano et al., 2012; Mozaffarian et al., 2015; Okabe and Miyamoto, 2018). According to an epidemiological study, 70–80% of all strokes are ischemic (Lo, 2010). Currently, recovering the blood flow to ischemic tissues is considered the most effective treatment for acute ischemic stroke (George et al., 2015; Powers et al., 2018). However, because of the narrow therapy window, the treatment of stroke still faces enormous difficulties (Saver et al., 2013). The pathophysiological mechanisms of cerebral ischemia/reperfusion (I/R) injury are complex (Durukan and Tatlisumak, 2007; Zheng et al., 2018), and include the formation of reactive oxygen species, glutamate excitotoxicity, intracellular Ca^{2+} overload and the inflammatory response (Eltzschig and Eckle, 2011; Li et al., 2011). These mechanisms have not been fully elucidated; however, they ultimately lead to neuronal apoptosis (Broughton et al., 2009; Tao et al., 2014). The inflammatory response to cerebral I/R injury has received attention in recent years and is now considered as a significant cause of neuronal apoptosis (Terao et al., 2008; Kim et al., 2014). The activation of nuclear factor kappa B signaling always induces the inflammatory response after cerebral ischemia injury (Bai et al., 2015), and regulates cell survival and the release of pro-inflammatory cytokines and enzymes (Baldwin, 2001). Therefore, searching for novel therapeutic targets that inhibit neuronal apoptosis rather than clot dissolution could be a potential strategy for acute ischemic stroke.

Epithelial-specific ETS-1 (ESE1) is a novel ETS transcription factor family member that is present in several organs, including small intestine, colon, prostate, kidney, liver, pancreas, and brain. (Oettgen et al., 1997; Longoni et al., 2013; Feng et al., 2016; Sinh et al., 2017). ETS transcription factor family members are involved in the processes of growth and development, including cell proliferation, differentiation, migration, and apoptosis (Yi et al., 2007; Garrett-Sinha, 2013). After the stimulation of inflammation or stress, the level of ESE1 is elevated in various cell types, which activates the nuclear factor kappa B pathway to regulate the transcription of target genes, such as nitric oxide synthase 2, ring oxidase (PTGS2/COX2), and angiogenin-1 (Oliver et al., 2012). Feng et al. (2016) found that ESE1 promotes neuronal apoptosis by activating nuclear factor kappa B in *in vivo* and *in vitro* neuro-inflammation models. ESE1 may, therefore, exert a significant role in regulating cell apoptosis in nervous system disease. However, the function of ESE1 in ischemic stroke is still uncertain.

Here, we mainly explored the possible function of ESE1 after cerebral I/R injury. In pilot experiments, we showed that ESE1 expression is increased in a rat cerebral ischemia/reperfusion model. We speculate that ESE1 expression strongly correlates with neuronal apoptosis after cerebral I/R.

Materials and Methods

Animals and experimental groups

Sixty adult male Sprague-Dawley rats (250–280 g) were purchased from the Animal Experiment Center of Comparative Medical Center of Yangzhou University, China (SCXK (Su)2012-0004). Rats were housed in an appropriate room at 25 ± 2°C and 45–55% humidity with a 12-hour light/dark cycle. All experimental procedures and protocols were performed in accordance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011), and were approved by the Institutional Animal Care and Use Committee of Yangzhou University, China on February 9, 2015 (approval number: YIACUC-15-0013).

Rats were randomly separated into two groups: (I) sham group (n = 9) and (II) I/R group (n = 45). In the I/R group, the rats were assigned to several subgroups according to different reperfusion time points (6, 12, 24, 48, and 72 hours).

Establishment of a global cerebral I/R model

The four-vessel occlusion method was applied as previously described (Lu et al., 2016). Briefly, after anesthesia with 10% chloral hydrate by intraperitoneal injection (300 mg/kg), a small incision was made in the neck skin. Bilateral vertebral arteries were electrocauterized and bilateral common carotid arteries were carefully exposed, and loosely ligated. The incision was sutured, and the rat returned to its cage. Twenty-four hours later, rats were anesthetized using 10% isoflurane. Common carotid arteries were re-exposed and occluded with aneurysm clips to cause global ischemia. After 20 minutes, the clips were released, and blood flow restored. For subsequent experiments, we selected the rats in which the eyes became white, the righting reflex was lost within 30 seconds with accompanying pupil dilation. The sham group received the same operation with the exception that the common carotid arteries were not occluded and the vertebral arteries were not cauterized. The rats were closely monitored and the body was kept at 36.5–37.5°C by an electric blanket.

PC12 cell culture and treatments

The rat pheochromocytoma cell line, PC12, was obtained from the Cell Resource Center of Yangzhou University, China. The cryopreserved PC12 cells were resuscitated and seeded in culture dishes with Dulbecco's modified Eagle's medium (Gibco, Suzhou, China), containing 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) (Xiao et al., 2012). The cells were cultured in an incubator with 5% CO_2 at 37°C and the medium was replaced every 2 days. Cells were treated with 0.25% trypsin (Gibco, Grand Island, NY, USA) and seeded into culture dishes at 7.5×10^4 cells/cm^2 for subsequent experiments. According to previous methods (Guo et al., 2013), chemical hypoxia was induced by adding cobalt chloride ($CoCl_2$; Sigma, St. Louis, MO, USA) at 250 μM to the medium. Cells were treated with $CoCl_2$ for different times (1, 3, 6, 12 and 24 hours). The PC12 cells cultured in normal condition were assigned to control group. Cell proteins were collected for analysis.

Small interference RNA (siRNA) transfection

PC12 cells were plated on 6-well culture plates (4×10^5 cells/

well) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After 2 hours, the medium was replaced with serum-free medium. siRNAs and a non-specific sequence RNA were purchased from GenePharma (Shanghai, China) and transfected using lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The siRNA sequences are listed in **Table 1**. Six hours after transfection, the medium was replaced and experiments performed 48 hours later. The silencing effect was verified by western blot assays and the calculation of ESE1 relative density.

Cytotoxicity test

The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Solarbio Technology, Beijing, China) test was used to detect cell viability at after 12 hours of hypoxia. Briefly, normal and transfected PC12 cells were plated in 96-well plates and incubated with $CoCl_2$ for 12 hours. After removing the medium, the MTT assay was conducted according to the manufacturer's protocol. The absorbance values were determined at 490 nm with a microplate reader (SpectraMax 190, Molecular Device, Sunnyvale, CA, USA). The percentage absorbance relative to the control was calculated to represent cell viability.

Passive avoidance test

A passive avoidance test was used to evaluate learning and memory ability according to a previous study (Azadbakht et al., 2015) at different reperfusion time-points (6, 12, 24, 48, and 72 hours). The equipment was composed of two separate compartments, one was light and the other dark. The floor consisted of stainless steel grids for delivering electric stimulation by an isolated stimulator. Rats were acclimatized in the equipment for at least 30 minutes before being subjected to passive avoidance experiments. Each rat was individually put into the light compartment for 10 seconds with the sliding door open. Because of the rats' preference for dark over light environments, they quickly entered the dark compartment. The door was promptly closed after the rats entered the dark compartment. An electric shock (50 Hz, 0.2 mA intensity; BPT Co., Tehran, Iran) was given to the mesh frame for 5 seconds. The rats were kept in the dark compartment for 20 seconds and were then returned to their home cage. Twenty-four hours after electric stimulation training, a memory retention test was conducted. Rats were put back in the light compartment with the sliding door open, but no electric shock was given, and the step-through latency was recorded.

Immunohistochemistry staining

ESE1 at 24 hours after reperfusion and in the sham group was examined by immunohistochemistry staining, as previously described (Feng et al., 2016). Rats were anesthetized with 10% chloral hydrate (300 mg/kg), and perfused through the heart with 0.9 % saline until the liver turned white, followed by 4% paraformaldehyde until the limbs were stiff. The brains were quickly and carefully removed and fixed in 4% paraformaldehyde at 4°C overnight, then dehydrated with 30% sucrose solution at 4°C until they sank. The brain was embedded in optimal cutting temperature medium and cut into coronal sections (5 μm) with a microtome (Leica CM1900, Beijing, China). The brain sections were washed with phosphate buffered saline for 5 minutes and then placed in a porcelain jar with trisodium citrate buffer. The porcelain jar was boiled in a pressure cooker for 3 minutes for antigen retrieval. The slices were washed with phosphate buffered saline three times for 5 minutes and incubated with 3% hydrogen peroxide for 10 minutes, and then washed again. The slices were blocked with 1% bovine serum albumin for 1.5 hours and incubated with primary monoclonal rat antibody-ESE1 (rabbit; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were then treated with the peroxidase anti-peroxidase method (Santa Cruz Biotechnology) for 60 minutes and visualized with diaminobenzidine. Sections were then gradually dehydrated in an ethanol gradient (70%, 80%, 95%, and 100% for 2 minutes each) and fixed with xylene. Slices were viewed through an inverted phase contrast microscope (K11027; Nikon, Tokyo, Japan).

Western blot assay

At different reperfusion times after cerebral I/R injury, ischemic tissues were collected for protein extraction. Briefly, the brains were removed and the hippocampal CA1 region in the ipsilateral infarct area was quickly dissected. Brain lysates were centrifuged at 10,000 × *g* for 30 minutes at 4°C and then the supernatant was collected. Tissue and cultured cell proteins were extracted with a protein extraction kit (Beyotime Biotech). Protein samples were electrophoresed and transferred to polyvinylidene difluoride membranes. The membrane was blocked with 5% fat-free milk at room temperature for 2 hours, and incubated with primary monoclonal rat antibodies as follows: ESE1 antibody (mouse, 1:500; Santa Cruz Biotechnology), active caspase-3 (rabbit, 1:1000; Cell Signaling, Beverly, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit, 1:1000; Santa Cruz Biotechnology), phosphorylated p65 (p-p65) (rabbit, 1:1000; Santa Cruz Biotechnology), at 4°C overnight. Next day, horse anti-mouse or goat anti-rabbit conjugated with horseradish peroxidase (1:1000, Cell Signaling) was added and incubated for 2 hours at room temperature, followed by visualization with an enhanced chemiluminescence system (Pierce, CO, USA). The gray value ratio of protein bands was quantified using ImageJ 1.37v software (NIH, Bethesda, MD, USA), with GAPDH as an internal control.

Immunofluorescence staining

Immunofluorescence staining was conducted according to previous methods (Li et al., 2015b) at 24 hours after reperfusion. Briefly, hippocampal sections (5 μm thick) were mounted onto glass slides, washed with phosphate buffered saline for 5 minutes and placed in a porcelain jar with trisodium citrate buffer. The porcelain jar was boiled in a pressure cooker for 3 minutes for antigen retrieval. The sections were treated with 0.1% Triton X-100 for 30 minutes and blocked with 10%

donkey serum for 2 hours. The sections were incubated with a primary monoclonal antibody against ESE1 (mouse, 1:500, Santa Cruz Biotechnology) and antibodies against glial fibrillary acidic protein (GFAP) (rabbit, 1:200; Santa Cruz Biotechnology) or NeuN (rabbit, 1:100; Santa Cruz Biotechnology) or Iba1 (rabbit, 1:150; Santa Cruz Biotechnology) at 4°C overnight, then incubated with goat anti-mouse Alexa-594 (1:500; Invitrogen, Carlsbad, CA, USA) or goat anti-rabbit Alexa-488 (1:500; Invitrogen). Immunofluorescence was viewed with a fluorescence microscope (LSM780, Zeiss, Germany). The immunofluorescence intensity for each rat was calculated from the average integrated optical density of all the acquired photos. The ESE1 co-localization rate was calculated by the overlap ratio of red fluorescence to green fluorescence using ImageJ 1.37v software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± SEM. Differences between two groups were determined with Student's *t* test. Differences among groups were compared with one-way analysis of variance followed by Tukey's *post hoc* test when there was a significant difference between groups. $P < 0.05$ was considered statistically significant.

Results

Learning and memory performance is decreased after cerebral I/R injury

Sixty mice were initially used, but six died before the experimental endpoint. As an important structure of learning and memory, the hippocampus is extremely sensitive to ischemia, and hippocampal neurons are severely damaged and cognition affected after cerebral I/R. In the passive avoidance test, the time avoiding the dark chamber was significantly shorter in the I/R group than in the sham group (**Figure 1**; $P < 0.05$). Meanwhile, no obvious difference among the different reperfusion time groups ($P > 0.05$) was observed. Learning and memory ability was significantly reduced after I/R injury.

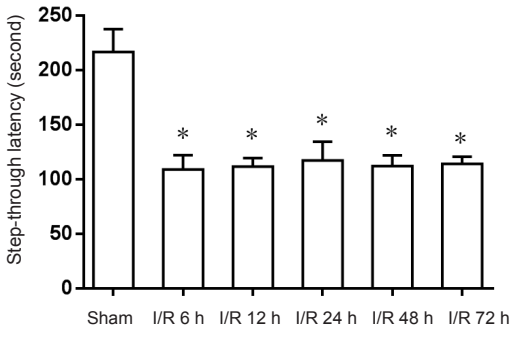

Figure 1 Learning and memory performance decreases after cerebral I/R injury.
Rats were evaluated using a passive avoidance test at different reperfusion time points. Compared with the sham group, the step-through latency of rats was significantly shortened in the I/R groups at all time points. *$P < 0.05$, *vs.* sham group. Data are expressed as the mean ± SEM ($n = 6$; one-way analysis of variance followed by Tukey's *post hoc* test). I/R: Ischemia/reperfusion; h: hours.

ESE1 and p-p65 levels in the hippocampus after cerebral I/R injury

Western blot analysis showed that the level of ESE1 increased from 6 hours after cerebral I/R and reached a peak at 24–48 hours ($P < 0.05$). It decreased thereafter but was still higher than normal (**Figure 2**). Meanwhile, p-p65 expression was also significantly increased after cerebral I/R, and the trend correlated with that of ESE1.

Distribution of ESE1 after cerebral I/R injury

Immunohistochemistry demonstrated that the number of ESE1-positive cells in the hippocampus of the I/R 24-hour group was significantly increased compared with that in the sham group (**Figure 3**). This result is consistent with the western blot analysis, indicating that ESE1 may be involved in cerebral ischemia injury.

ESE1 mainly exists in hippocampal neurons after cerebral I/R injury

To identify the cell types expressing ESE1, immunohistochemistry was performed. In hippocampal sections of the I/R 24-hour group, ESE1 was mainly located in neurons (**Figure 4A–C** and **J**), rather than astrocytes (**Figure 4D–F** and **J**) and microglia (**Figure 4G–I** and **J**).

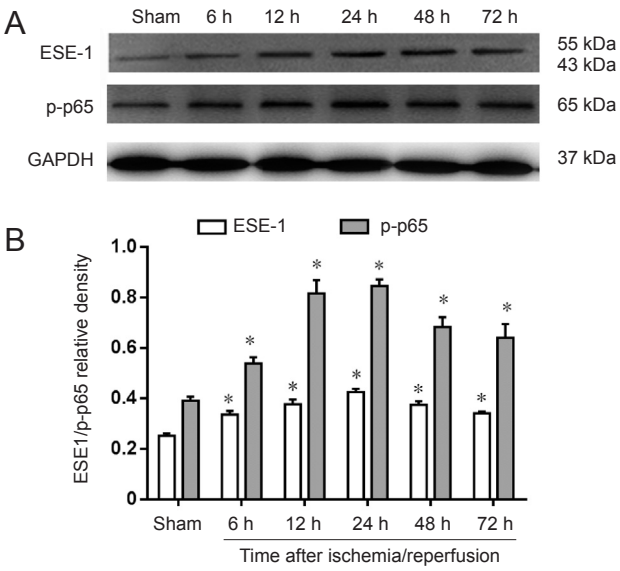

Figure 2 Expression of ESE1 and p-p65 in the hippocampal CA1 region after cerebral ischemia/reperfusion injury.
After different reperfusion time points, total protein was extracted for western blot analysis of ESE1 and p-p65. The gray value ratio of protein bands was quantified by ImageJ software, with GAPDH as the internal control. (A) Western blot assay shows ESE1 and p-p65 expression after cerebral ischemia/reperfusion injury at different time points. (B) Quantitative analysis revealed the variation in levels of ESE1 and p-p65 after cerebral ischemia/reperfusion injury. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by Tukey's *post hoc* test). *$P < 0.05$, *vs.* sham group. ESE1: Epithelial-specific ETS-1; p-p65: phosphorylated p65; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; h: hours.

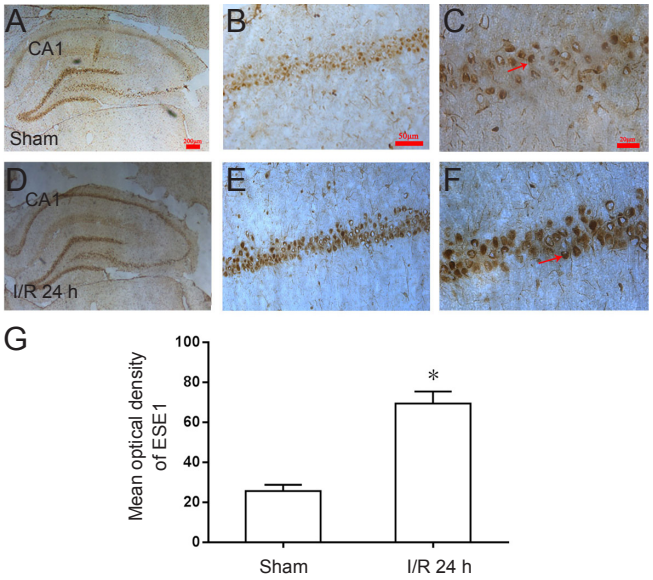

Figure 3 Average integrated optical density of ESE1 increases in the CA1 region after cerebral I/R.
(A–F) Immunohistochemistry staining of ESE1 in the hippocampal CA1 region in the sham group (A–C) and I/R 24-hour group viewed through an inverted phase contrast microscope (D–F). The red arrows indicate ESE1-positive cells. (G) Quantitative analysis of the average integrated optical density of ESE1 in the CA1 region. $*P < 0.05$, *vs.* sham group. Data are expressed as the mean ± SEM ($n = 3$; Student's *t* test). Scale bars: 200 μm (A, D), 50 μm (B, E), 20 μm (C, F). I/R: Ischemia/reperfusion; ESE1: epithelial-specific ETS-1; h: hours.

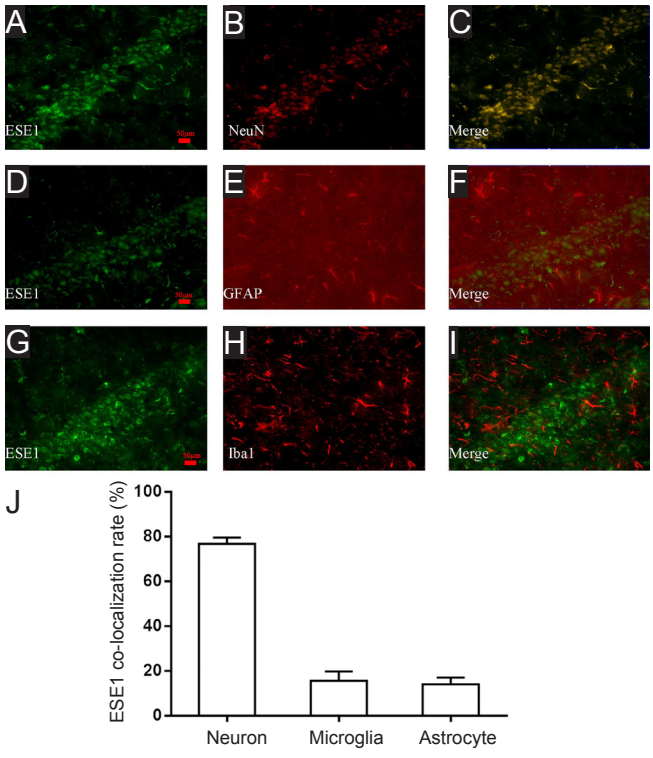

Figure 4 Immunofluorescence staining shows that ESE1 mainly exists in hippocampal neurons after cerebral ischemia/reperfusion injury.
(A–I) Double immunofluorescence staining of ESE1 (green, A, D, G) with NeuN (red, B, neuron), GFAP (red, E, astrocyte) or Iba1 (red, H, microglia) in rat hippocampus at 24 hours after reperfusion. The yellow signal in the merged images indicates co-localization of ESE1 with the neuronal marker. (J) Quantitative analysis of the ESE1 co-localization rate (%) in the CA1 region. Data are expressed as the mean ± SEM ($n = 3$). Scale bars: 50 μm. GFAP: Glial fibrillary acidic protein; ESE1: epithelial-specific ETS-1.

ESE1 associates with neuronal apoptosis after cerebral I/R injury

Neurons are the most sensitive cells in the brain to I/R damage, especially pyramidal neurons in the hippocampal CA1 region (Mitani et al., 1992; White et al., 2000). To determine whether levels of ESE1 are related to neuronal apoptosis after brain ischemia, levels of the apoptosis index molecule, active caspase-3, in hippocampal tissues were detected by western blot assays in I/R and sham groups. Levels of active caspase-3 were low, and then increased at 6 hours after reperfusion in the sham group. After a gradual rise, the peak was observed at 48 hours and then levels gradually declined (**Figure 5A** and **B**), which was similar to the profile of ESE1 levels (**Figure 2A**). Meanwhile, at 24 hours after reperfusion, ESE1 and active caspase-3 were co-expressed in hippocampal neurons (**Figure 5C–E**). These results indicate that ESE1 is associated with neuronal apoptosis after cerebral I/R injury.

Changes in ESE1, p-p65 and active caspase-3 levels in hypoxia models

To further determine ESE1 function in cerebral ischemia, an *in vitro* chemical hypoxia model, induced by $CoCl_2$, was constructed in PC12 cells (Yang et al., 2011). The $CoCl_2$ concentration was chosen as 250 μM because cell activity was inhibited to the greatest extent at this concentration (Xiao et al., 2012; Guo et al., 2013). The active caspase-3 level be-

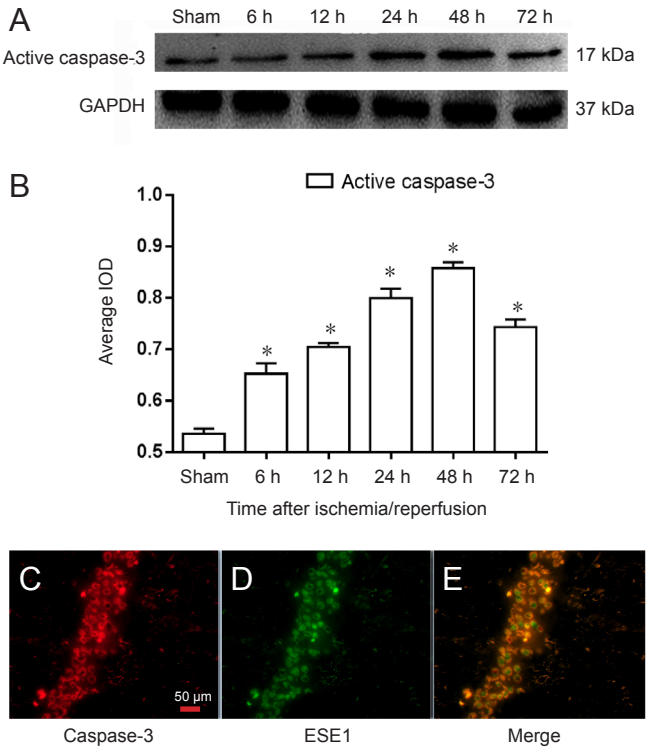

Figure 5 ESE1 associates with neuronal apoptosis in the hippocampal CA1 region after cerebral ischemia/reperfusion injury.
(A) Western blot assay of active caspase-3 levels in the hippocampal CA1 region. (B) Quantitative analysis of average integrated optical density (IOD) of active caspase-3. $*P < 0.05$, *vs.* sham group. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by Tukey's *post hoc* test). (C–E) Double immunofluorescence staining: The co-expression of caspase-3 (red) and ESE1 (green) was viewed through a fluorescence microscope. Scale bar: 50 μm. ESE1: Epithelial-specific ETS-1; h: hours.

gan to rise at 1 hour after $CoCl_2$ stimulation, and reached a peak at 6–12 hours (**Figure 6**), which meant the cell hypoxia model was successfully established. In addition, levels of ESE1 and p-p65 increased during apoptosis, and reached a peak at 12 hours, then gradually declined, but remained above normal levels ($P < 0.05$). From the above results, we suggest that ESE1 participates in neuronal apoptosis *in vitro* by activating nuclear factor kappa B expression.

Inhibition of ESE1 expression alleviates neuronal apoptosis induced by $CoCl_2$ *in vitro*

For a more detailed understanding of ESE1 function in cerebral I/R and to verify our assumption, three ESE1 siRNAs were established (**Table 1**) to knock down ESE1 in PC12 cells. Western blot analysis showed that siRNA-ESE1, especially siESE1#3, strongly reduced ESE1 abundance (**Figure 7A** and **B**). MTT assays demonstrated that cell viability was remarkably lower in the $CoCl_2$ group than in the control group (**Figure 7E**). Consistent with the western blot results, knock-down of ESE1, especially siESE1#3, increased PC12 cell viability compared with the $CoCl_2$ group (**Figure 7E**). Meanwhile, compared with the control and non-specific siRNA groups, ESE1 knockdown resulted in significant down-regulation of active caspase-3 and p-p65 levels in PC12 cells stimulated by $CoCl_2$ (**Figure 7C** and **D**), indicating that ESE1 inhibition might alleviate neuronal apoptosis.

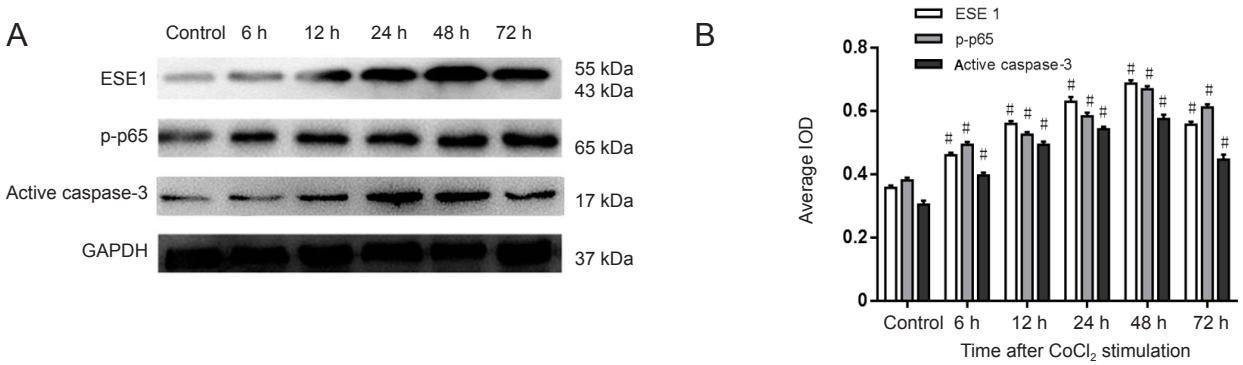

Figure 6 Changes of ESE1, p-p65 and active caspase-3 expression in an *in vitro* hypoxic model.
(A) ESE1, active caspase-3 and p-p65 expression were increased in PC12 cells after hypoxia induced by 250 μM $CoCl_2$. (B) Quantitative analyses of the average integrated optical density (IOD) of ESE1, active caspase-3 and p-p65. #$P < 0.05$, *vs.* control group. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by Tukey's *post hoc* test). ESE1: Epithelial-specific ETS-1; p-p65: phosphorylated p65; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; h: hour(s).

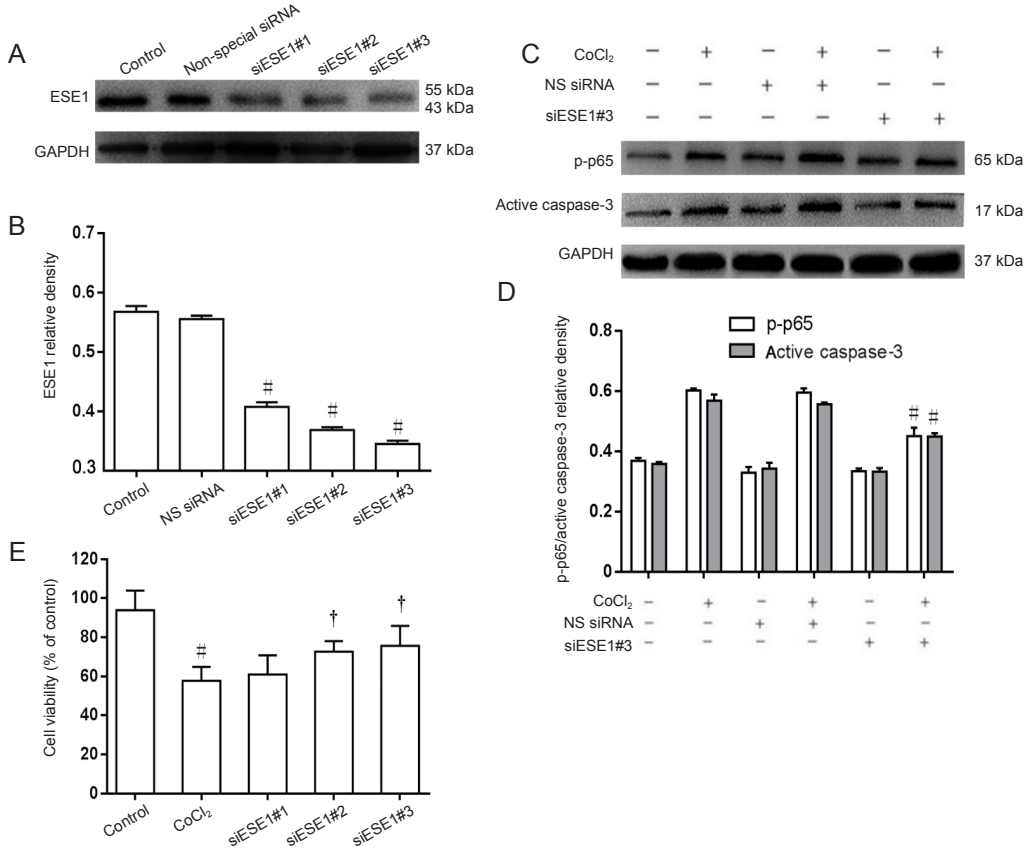

Figure 7 Inhibition of ESE1 expression alleviates neuronal apoptosis induced by $CoCl_2$ *in vitro*.
(A, B) Western blot assay for the effect of silencing ESE1 by siRNA: ESE1 expression could be knocked-down by ESE1 siRNA sequences 1, 2 and 3, with siESE1#3 having the strongest effect ($P < 0.05$). (C, D) ESE1 expression after transfection with NS siRNA (50 nM) or siESE1#3 (50 nM) in PC12 cells treated with or without $CoCl_2$ (250 μM). After hypoxia induced by $CoCl_2$, ESE1 expression was knocked-down and levels of p-p65 and active caspase-3 were significantly reduced ($P < 0.05$). (E) MTT assay for cell viability in each group after $CoCl_2$ induction. #$P < 0.05$, *vs.* control group; †$P < 0.05$, *vs.* $CoCl_2$ group. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by Tukey's *post hoc* test). ESE1: Epithelial-specific ETS-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; p-p65: phosphorylated p65; con: control.

Table 1 Primer sequences

siRNA	Sequence (5′–3′)
siRNA1	Positive-sense strand: GGA GCT GCG ACT AGT CTT T
	Antisense strand: CCT CGA CGC TGA TCA GAA A
siRNA2	Positive-sense strand: CCT CAG ACA AGA TCC CAA A
	Antisense strand: GGA GTC TGT TCT AGG GTT T
siRNA3	Positive-sense strand: CCC AGG TAC TGG ATT GGA T
	Antisense strand: GGG TCC ATG ACC TAA CCT A

siRNA: Small interference RNA.

Discussion

This study explored the role of ESE1 in cerebral ischemia injury. The hippocampal CA1 region is the most sensitive brain region to ischemia, and is one of the most important structures for learning, memory and emotion. When brain ischemia occurs, it is likely to cause cognitive dysfunction, depression, epilepsy and other serious complications (Molinaro et al., 2011). Our results showed that learning and memory performance was weakened after cerebral I/R injury, which indicates damage to the hippocampus. We also showed that ESE1 levels were up-regulated in the hippocampus after ischemia, from 6 hours after cerebral I/R, peaking at 24–48 hours. Active caspase-3 increased after ischemia and also co-localized with ESE1 in hippocampal CA1 region neurons. In addition, we also confirmed that ESE1 is mainly distributed in neurons. Changes to ESE1 and active caspase-3 levels were similar in different I/R groups; both were elevated at 6 hours and peaked at 24–48 hours. ESE1 was also remarkably expressed in PC12 cells with chemical hypoxia induced by $CoCl_2$. Furthermore, after knockdown of ESE1 expression with siRNA, the levels of ESE1 and active caspase-3 decreased after $CoCl_2$ induction. Our findings also showed that p-p65 expression was similar to that of ESE1 in the *in vitro* and *in vivo* models. After knocking down ESE1, levels of p-p65 and active caspase-3 remarkably decreased. In conclusion, our data demonstrated that ESE1 might accelerate neuronal apoptosis after cerebral I/R injury *via* activation of nuclear factor kappa B expression.

The ETS family is one of the largest families of transcription factors and all its members contain a highly conserved DNA binding sequence (the Ets domain), that binds to enhancer or promoter sequences rich in purines (GGAA/T) of target genes (Maroulakou and Bowe, 2000; Yang et al., 2016). ESE1 is a new type of ETS family transcription factor. In addition to the Ets domain, it also has an AA/T hook domain (Findlay et al., 2013). ESE1 interacts with target genes through these two unique DNA binding domains to regulate the transcription of protein molecules in different physiological and pathological processes (Seidel et al., 2002; Seth and Watson, 2005). Although numerous previous studies show that ESE1 is only expressed in epithelial-rich tissues (Oettgen et al., 1997), many experiments have also showed that ESE1 expression can be induced by inflammatory stimuli in various cell types of non-epithelial origin, such as vascular endothelial cells (Grall et al., 2003), monocytes/macrophages (Grall et al., 2005), endothelial cells (Zhan et al., 2010) and neurons (Feng et al., 2016). Many studies have demonstrated its therapeutic potential in different diseases. Li et al. (2015a) revealed that in ulcerative colitis, ESE1 regulates the apoptosis of intestinal epithelial cells by activating the nuclear factor kappa B pathway. Furthermore, inhibiting ESE1 expression in colorectal cancer reduced the proliferation of tumor cells and accelerated cell apoptosis (Wang and Chen, 2014). Ischemic injury occurs in a sterile environment and involves the innate and adaptive immune systems (Gesuete et al., 2014) and ESE1 also participates in regulating the development of immune cells (Gallant et al., 2004). Feng et al. (2016) first revealed that ESE1 promoted neuronal apoptosis in neuroinflammation by up-regulating nuclear factor kappa B activation. Their study indicated that ESE1 may be associated with neuronal apoptosis in neurological diseases. Our study further found that ESE1 abundance was increased and is involved in neuronal apoptosis after cerebral ischemia injury, while knocking down ESE1 decreased apoptosis of hypoxic PC12 cells.

The pathological process that ESE1 regulates in neuronal apoptosis after cerebral I/R injury remains to be explored. In ischemic stroke, restoration of blood flow and reoxygenation is always accompanied by deterioration injury and a deeper inflammatory response, which is named I/R injury (Eltzschig et al., 2011; Dong et al., 2013). The genes involved in apoptosis can be divided into two categories: one is pro-apoptotic and includes genes such as Bax, c-jun, c-fos and p53, and the other is anti-apoptotic and includes genes such as Bcl2 (Longley and Allen, 2004; Tan and Fu, 2005; Roset and Ortet, 2007). Caspase-3 is the most crucial apoptotic member of the caspase family and active caspase-3 can induce irreversible injury (Mirzayans et al., 2016). Hu et al. (2015) found that apoptotic cell numbers were significantly increased after focal cerebral I/R injury in caspase-3 overexpressing mice. Therefore, reducing caspase-3 activation alleviates brain damage after cerebral ischemia. This study confirmed that ESE1 was mainly expressed in neurons in the hippocampal CA1 region after cerebral ischemia injury. Moreover, active caspase-3 was upregulated after ischemia. We also detected that ESE1 was tightly associated with active caspase-3 expression in neurons after brain ischemia. ESE1 inhibition decreased active caspase-3 levels *in vitro*.

The post-inflammatory cascade plays a vital role in cerebral I/R injury (Shichita et al., 2012; Zhang et al., 2018), eventually causing neuronal apoptosis (Broughton et al., 2009). Nuclear factor kappa B is a major inflammatory regulatory factor and exists in the cytoplasm in an inactive form in the resting state. When stimulated, the nuclear factor kappa B subunit, p65, can be phosphorylated to the active form (Gorina et al., 2011; Molinaro et al., 2011). Grall et al. (2003) found that after stimulation by pro-inflammatory cytokines, the p65 subunit translocated to the cell nucleus and further transactivated the ESE1 promoter *via* a high-affinity nuclear factor kappa B binding site to increase ESE1 expression. Machado et al. (2012) have shown that restraining nuclear factor kappa B activity or knocking out p50 in a cerebral

ischemia model reduced infarct size and neuronal apoptosis. Therefore, we predicted that ESE1 regulates neuronal apoptosis *via* nuclear factor kappa B after cerebral I/R injury. In this study, p-p65 expression was obviously increased after ischemia *in vivo* and $CoCl_2$-induced hypoxia *in vitro*. Treatment with siRNA-ESE1 significantly down-regulated p-p65 levels.

In conclusion, our study emphasizes the significant role of ESE1 in neuronal apoptosis after cerebral I/R injury. ESE1 was upregulated after ischemia, and ESE1 siRNA knockdown significantly reduced neuronal apoptosis by inhibiting nuclear factor kappa B activity. Due to limited funding, there are some deficiencies in this study. We did not interfere with ESE1 expression *in vivo* to explore the detailed molecular mechanisms of neuronal apoptosis after cerebral ischemia. Further research is needed to construct a global ESE1 knock-out model, to culture primary hippocampal neurons and to further explore the molecular mechanism of cerebral I/R injury. Thus, our findings provide a promising target for future neuroprotection therapies after cerebral I/R injury.

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