

Full-length article

Neuroprotective effects of scutellarin on rat neuronal damage induced by cerebral ischemia/reperfusion

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Key words

scutellarin; brain ischemia; nitric-oxide synthase; vascular endothelial growth factor; basic fibroblast growth factor

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Abstract

Aim: To investigate the neuroprotective effect and mechanisms of scutellarin, a flavonoid extracted from *Erigeron breviscapus* Hand Mazz, against neuronal damage following cerebral ischemia/reperfusion. **Methods:** Rats were pretreated ig with scutellarin for 7 d and then subjected to cerebral ischemia/reperfusion (I/R) injury induced by a middle cerebral artery occlusion (MCAO). The infarct volume and neurological deficit were determined by TTC staining and Longa's score. The permeability of the blood-brain barrier was evaluated by measurement of the Evans blue (EB) content in the brain with a spectrophotometer. The total NOx content was determined. Nitric oxide synthase (NOS) isoforms (iNOS, eNOS, nNOS) and the key angiogenic molecules, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were detected by Western blotting. **Results:** Scutellarin significantly reduced infarct volume ($P < 0.05$ or $P < 0.01$), ameliorated the neurological deficit and reduced the permeability of the blood-brain barrier (BBB) ($P < 0.05$). When rats were pretreated with scutellarin (50 or 75 mg/kg), upregulation of eNOS expression and downregulation of VEGF, bFGF, and iNOS expression was observed, whereas scutellarin had no effect on nNOS expression. **Conclusion:** Scutellarin has protective effects for cerebral injury through regulating the expression of NOS isoforms and angiogenic molecules.

Introduction

Cerebral ischemia is accompanied by a marked inflammatory process, which is initiated by higher levels of expression of cytokines, adhesion molecules, and other inflammatory mediators, including nitric oxide^[1,2]. Recent studies have demonstrated that nitric oxide (NO) and pro-inflammatory cytokines released by microglial cells, which act as resident macrophage-like cells in the brain, are partly responsible for neuronal cell death. NO levels are associated directly with the development of brain injury in strokes and other neuro-pathological disorders in humans^[3,4].

Following acute ischemic or hypoxic injury to the brain, over-entry of Ca²⁺ into cells causes the activation of nitric oxide synthase (NOS), which catalyzes an enzymatic reaction, leading to the synthesis of nitric oxide^[5–7]. Three kinds of distinct NOS isoforms have been identified, including neu-

ronal nNOS, endothelial eNOS, and an inducible isoform, iNOS, originally isolated from macrophages. nNOS and eNOS are constitutively expressed and calcium-dependent, whereas iNOS is expressed in response to various inflammatory stimuli, and its activity is independent of intracellular calcium concentrations. NO can be neuroprotective or neurotoxic during cerebral ischemia, depending on the NOS isoform involved. eNOS produces NO with beneficial effects (vasodilation, inhibition of platelet aggregation and polymorphonuclear neutrophil adhesion)^[7–9], whereas NO overproduction by nNOS or iNOS during ischemia is cytotoxic. Based on these findings, it thought that the NO-synthases could be attractive targets for treating cerebral ischemia-induced neuronal damage^[10,11].

Scutellarin, a flavonoid, is the major active ingredient extracted from *Erigeron breviscapus* Hand Mazz, a plant used in Chinese herbal medicine, which is a Ca²⁺-channel-block-

ing agent used for the clinical treatment of cerebrovascular disorders. Studies have demonstrated the protective effects of scutellarin on brain injury induced by cerebral ischemia/reperfusion (I/R) through interaction with a wide variety of targets because of its anti-oxidative and anti-inflammatory actions, and its ability to attenuate neuronal damage^[12,13]. It is presumed that these effects might be related to the effects of scutellarin on the NO synthases. Therefore, the objective of the present study was to elucidate the effects of scutellarin on the expression of the NOS isoforms (iNOS, eNOS, nNOS) in a model of cerebral I/R in rats. Moreover, the key angiogenic molecules, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were also studied.

Materials and methods

Chemicals and drugs Scutellarin was supplied by Yuxi Pharmaceuticals (Kunming, China). The purity of this compound was more than 96% and it was dissolved in saline before use. 2,3,5-Triphenyltetrazolium chloride (TTC, No 20010201) was obtained from the Shanghai Chemical Agent Company. All other chemicals and solvents were of analytical grade.

Animal treatment and administration Male Sprague-Dawley rats (Grade II, Certificate No 19-050), weighing 230–280 g, were obtained from the Experimental Animal Center of Tongji Medical College. Rats were housed at a constant temperature of 22 °C under a 12 h light-dark cycle with free access to food and drinking water. Rats were divided into 5 groups. The sham-operated and vehicle-treated I/R groups were pretreated with 0.5 mL/kg ig saline for 7 d before ischemia, and the scutellarin-treated I/R groups were pretreated with 25, 50, 75 mg/kg ig scutellarin for 7 d before ischemia.

Cerebral I/R procedure Rats were anesthetized with chloral hydrate (300 mg/kg, ip). Brain I/R injury was induced by a middle cerebral artery occlusion (MCAO) as described previously^[14]. Briefly, the right common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were isolated via a ventral midline incision. A 50 mm length of monofilament nylon suture (ϕ 0.22–0.24 mm), with its tip rounded by heating near a flame, was introduced into the ECA lumen and advanced into the ICA for a distance of 18–20 mm in order to block the origin of the MCA. The body temperature of the rats was maintained at 36.5–37.5 °C during the surgical procedure with an infra red heat lamp. Sham-operated animals were not exposed to I/R. After 2 h of ischemia, the nylon suture was withdrawn to establish reperfusion. After arousal from anesthesia, the rats were returned to the cages.

Behavioral testing and measurement of infarct area After 24 h reperfusion, the neurological deficit score of each rat

was obtained according to Longa's method^[14] by a single experimenter, who was blinded to the experimental treatment groups. The neurological findings were scored on a 5-point scale: no neurological deficit=0, failure to extend right paw fully=1, circling to right=2, falling to right=3, did not walk spontaneously and had depressed levels of consciousness=4. Then the rats were anesthetized with 10% chloral hydrate (350 mg/kg) ip and subsequently decapitated. The brains were removed for measurement of infarct volume by using the TTC staining method. Five thin sections were selected from the thick slices at 2 mm intervals (from the anterior 5 mm to the anterior 13 mm) to determine the infarct areas. The slices were immersed in 2% triphenyltetrazolium chloride in saline and incubated at 37 °C for 20 min, and then fixed with 10% formaldehyde (Sigma) neutral buffer solution (pH 7.4). At that time, the infarct tissue was unstained, whereas the normal part was stained red. Using a computerized image analysis system (NIH Image, Version 1.61), the infarct areas on each slice were summed and multiplied by slice thickness to give the infarct volume, and then expressed as the percentage of infarction per ipsilateral hemisphere.

Evaluation of permeability of blood-brain barrier The integrity of the blood-brain barrier (BBB) was investigated using Evans blue (EB) dye extravasation, according to the method of Matsuo *et al*^[15]. Briefly, after 6-h reperfusion, the rats were treated with EB dye (2% in saline, 3 mg/kg iv). After 45 min, the rats were anesthetized with 10% chloral hydrate (350 mg/kg ip) and then the rats' chests were subsequently opened. Physiological saline was perfused through the left ventricle until a colorless perfusion fluid was obtained from the right atrium. The cranial vault was opened, and the brain was removed, weighed (wet tissue) and placed in a 50% trichloroacetic acid solution. After homogenization and centrifugation, the supernatant (extracted dye) was diluted with ethanol (1:3) and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrometer (Hitachi, Tokyo, Japan). Calculations of the amount of EB dye in the tissue were based on a linear standard curve and were expressed per gram of tissue.

Determination of total NO_x content in brain tissue At the end of 2 h ischemia and 24 h reperfusion, the rats were decapitated and the ischemic hemispheres were removed for assay of the NO level in ischemic brain tissue. The levels of metabolic products (NO₂ and NO₃) *in vivo* were determined by using a chemiluminescent NO detector (Siever 280i) as described previously^[16].

Western blot analysis Western blot analysis was performed after 24 h reperfusion. The rats' brains were removed and the ischemic hemispheres were used for assay of the protein ex-

pression of iNOS, eNOS, nNOS, VEGF and bFGF. The hippocampus and the cortex were quickly isolated and rinsed in sterilized water on ice, and then stored at -80°C until use. Protein determination was performed according to the Lowry method. The obtained protein samples were subjected to 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis, using 7.5%–15% polyacrylamide gel, and electrotransferred to polyvinylidene difluoride filter (PVDF) membranes. To reduce non-specific binding, the PVDF was blocked for 2 h at room temperature with 5% non-fat milk in phosphate-buffered saline (PBS). Then membranes were incubated overnight at 4°C with the primary antibodies for iNOS (anti-rabbit iNOS mouse monoclonal antibody, 1:200 dilution, Santa Cruz), eNOS (anti-human eNOS rabbit polyclonal antibody, 1:200 dilution, Affinity Bioreagents), nNOS (anti-human nNOS rabbit polyclonal antibody, 1:200 dilution, Sanying Biotechnology), VEGF (antihuman VEGF rabbit polyclonal antibody, 1:200 dilution, Santa Cruz) or bFGF (anti-human bFGF rabbit polyclonal antibody, 1:500 dilution, Santa Cruz), respectively. After incubation with the antibodies, the membranes were washed with PBS-Tween-20 (PBS-T: 10 mmol/L phosphate buffer, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) for 30 min and incubated in the relevant horseradish peroxidase-conjugated secondary antibody (1:600 dilution) for 30 min. The membranes were washed again with PBS-T and immunoreactive protein bands were visualized using the enhanced chemiluminescence detection system.

Statistical analysis Data are expressed as mean \pm SD and analyzed by using Microsoft Excel 2002. Statistical analyses were performed by using Student's *t*-test. $P < 0.05$ was considered significant.

Results

Effects of scutellarin on the infarct area, neurological score and the permeability of the blood-brain barrier Scutellarin (50 or 75 mg/kg) significantly reduced the infarct area and ameliorated the neurological deficit ($P < 0.05$ or $P < 0.01$ vs vehicle-operated group) (Table 1). The EB content of brain tissue after I/R for sham-operated, vehicle-operated and scutellarin-operated groups (25, 50, or 75 mg/kg) was 3.83 ± 1.03 , 8.45 ± 1.67 , 7.45 ± 1.77 , 5.02 ± 1.12 , and 4.45 ± 1.05 , respectively (Figure 1). There was a significant increase in the permeability of the BBB in rats in the vehicle-treated group compared with the sham-treated group ($P < 0.01$). Scutellarin (50 or 75 mg/kg) obviously inhibited the increased EB content induced by cerebral I/R, and there was no obvious difference between the 2 doses.

Effects of scutellarin on total NOx production After cerebral I/R, total NOx production, as determined by NOx content in the ischemic brain hemispheres, was markedly in-

Table 1. Effects of scutellarin on the cerebral infarct area and neurological score after 2 h ischemia and 24-h reperfusion (I/R) in rats. $n=6$. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs vehicle-operated group (I/R+NS).

Groups	Dose/ $\text{mg}\cdot\text{kg}^{-1}$	Ratio of infarct area/%	Neurological score (on a 5-point scale)
Sham	0	–	–
Vehicle	0	31.26 ± 6.02	2.92 ± 1.27
I/R+scutellarin	25	25.63 ± 5.12	1.93 ± 0.84
	50	18.23 ± 3.63^b	1.32 ± 0.65^b
	75	9.24 ± 4.11^c	0.92 ± 0.64^c

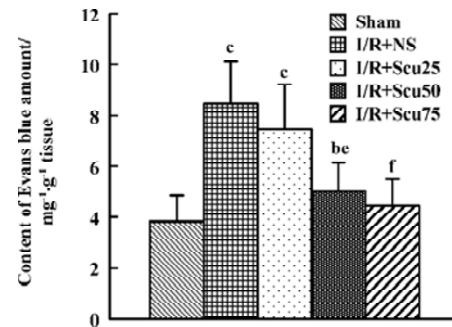


Figure 1. Effects of scutellarin on the blood-brain barrier after 2-h ischemia and 6-h reperfusion. See legend of Table 1 for rat treatments. Scu25, 50, and 75 correspond to 25, 50, and 75 mg/kg scutellarin, respectively. Mean \pm SD. $n=6$. ^b $P < 0.05$, ^c $P < 0.01$ vs sham. ^e $P < 0.05$, ^f $P < 0.01$ vs vehicle (I/R+NS).

creased in the vehicle rats (4.87 ± 0.90) compared with the sham-treated rats (1.83 ± 0.34) ($P < 0.01$, Figure 2). Total NOx production in rats pretreated with scutellarin at concentrations of 50 or 75 mg/kg (3.01 ± 0.68 , 2.31 ± 0.48), were significantly reduced compared with the vehicle-operated group ($P < 0.05$ or $P < 0.01$, respectively) (Figure 2).

After 24 h reperfusion, the expression levels of iNOS, eNOS and nNOS were detected in the hippocampus and in the cortex, with molecular masses of 130, 140, 160 kDa, respectively. In the vehicle-treated group, the expression levels of the 3 NO synthases in the hippocampus and in the cortex markedly increased after cerebral I/R ($P < 0.01$ or $P < 0.05$, Figure 3A, 3B, lane 2). Densitometric analysis showed that the protein levels of eNOS and iNOS in the scutellarin-treated (50 or 75 mg/kg) rats were $368.0 \pm 70.3\%$ and $278.0\% \pm 56.6\%$ in the hippocampus (Figure 3A, lanes 3, 4), and $198.1\% \pm 19.2\%$ and $148.3\% \pm 17.6\%$ in the cortex

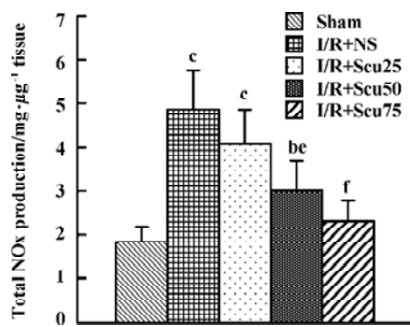


Figure 2. Effect of scutellarin on the total NOx production after 2 h ischemia and 24 h reperfusion. See legend of Table 1 for rat treatments. Scu25, 50, and 75 correspond to 25, 50, and 75 mg/kg scutellarin, respectively. *n*=6. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs sham. ^e*P*<0.05, ^f*P*<0.01 vs vehicle (I/R+NS). Effects of scutellarin on expression of the 3 NOS isoforms (iNOS, eNOS, nNOS) and the angiogenic molecules (VEGF and bFGF).

(Figure 3B, lane 3, 4) for iNOS; and 469.0%±40.5% and 523.0%±67.3% in the hippocampus (Figure 3A, lanes 3, 4), and 188.3%±31.2% and 234.2%±37.8% in the cortex (Figure 3B, lanes 3, 4) for eNOS, respectively. The data indicate that scutellarin downregulated the expression of iNOS and simultaneously upregulated that of eNOS as compared with the vehicle-operated group (*P*<0.05 or *P*<0.01, respectively), whereas there was no difference in nNOS expression in the hippocampus or the cortex between the vehicle-treated and scutellarin-treated rats.

Immunoblot analysis showed single bands with molecular masses of approximately 39 and 18 kDa, which correspond to VEGF and bFGF in the hippocampus and in the cortex, respectively. The bands obtained from the vehicle-operated I/R rats (VEGF: 278.2%±43.4% in the hippocampus and 256.7%±36.8% in the cortex; bFGF: 432.2%±50.4% in the hippocampus and 289.4%±49.7% in the cortex) (Figure 3C, 3D, lane 2) were stronger than those from the sham group rats (VEGF: 111.9%±24.2% in the hippocampus and 123.9%±16.1% in the cortex; bFGF: 98.8%±15.4% in the hippocampus and 98.8%±10.9% in the cortex) (Figure 3C, 3D, lane 1, *P*<0.01). Scutellarin at doses of 50 or 75 mg/kg (Figure 3C, 3D, lanes 3, 4) significantly decreased the expression of VEGF and bFGF in the hippocampus and in the cortex, as compared with the vehicle-operated group (*P*<0.05 or *P*<0.01). When rats were pretreated with scutellarin at a concentration of 50 or 100 mg/kg, the VEGF protein levels in the hippocampus were 189.8%±35.4% and 123.5%±30.1%, whereas in the cortex they were 178.2%±22.1% and 145.7%±11.9%, respectively; bFGF protein levels in the hippocampus were 212.9%±33.2% and 134.5%±19.1%, while in the cortex they were 212.9%±30.4% and 145.6%±17.6%, respectively.

Discussion

In the present study, we demonstrated that scutellarin (at doses of 50 or 75 mg/kg) significantly reduced infarct volume, ameliorated the neurological deficit and reduced the permeability of the BBB after cerebral I/R. Therefore, the conclusions obtained from the above observations were that scutellarin has protective effects for the neuronal damage induced by cerebral I/R in rats.

Evidence has accumulated that NO produced both before and after cerebral ischemia may be an important factor in the pathogenesis of neuronal ischemic injury. NO is a signaling molecule that regulates many biological processes in the brain. The present paper also investigated the effects of scutellarin on the total NOx content in rat brain tissues after cerebral I/R. Our results showed that total NOx production, as determined by NOx content, in the ischemic brain hemispheres was markedly increased in cerebral I/R rats, which indicates that NO regulates the severity of cerebral ischemic injury. However, NOx content markedly decreased in brain tissues after treatment with scutellarin at doses of 50 or 75 mg/kg.

Numerous studies have been conducted regarding the differential roles of NOS isoforms and their temporal NO production in the pathogenesis of ischemic brain injury^[17-19]. eNOS-derived NO is thought to be beneficial for promoting collateral circulation and microvascular flow, whereas nNOS- and iNOS-derived NO is detrimental in the ischemic brain. NO is a nontoxic agent and acts as a second messenger in normal brain; however, in the presence of O₂⁻, NO reacts with O₂⁻ to form ONOO⁻ or nitrogen dioxide (NO₂⁻), causing injury to the mitochondrial electron transport system, resulting in neuronal damage. In addition, excess NO stimulates ADP ribosyltransferase and binds closely to iron-sulfur centers of enzymes, including enzymes involved in the mitochondrial electron transport chain and the tricarboxylic acid cycle (TCA), and DNA^[20-22]. In view of the detrimental and beneficial roles of NOS isoforms in ischemic brain injury, further investigations into the effect of scutellarin on the expression of NOS isoforms (iNOS, eNOS, nNOS) both in the hippocampus and in the cortex after cerebral I/R were also performed in the present study. We found that expression of the NO-synthases in the hippocampus and cortex all markedly increased after cerebral I/R in the vehicle-operated group, a similar finding to those of previously studies^[22-26]. Scutellarin at doses of 50 and 75 mg/kg downregulated iNOS expression and upregulated eNOS expression, which partly account for its protective effect on brain damage induced by cerebral I/R.

Increasing evidence has shown that some angiogenic molecules, including VEGF and bFGF, increase in concentration after cerebral I/R. VEGF is an angiogenesis and vascu-

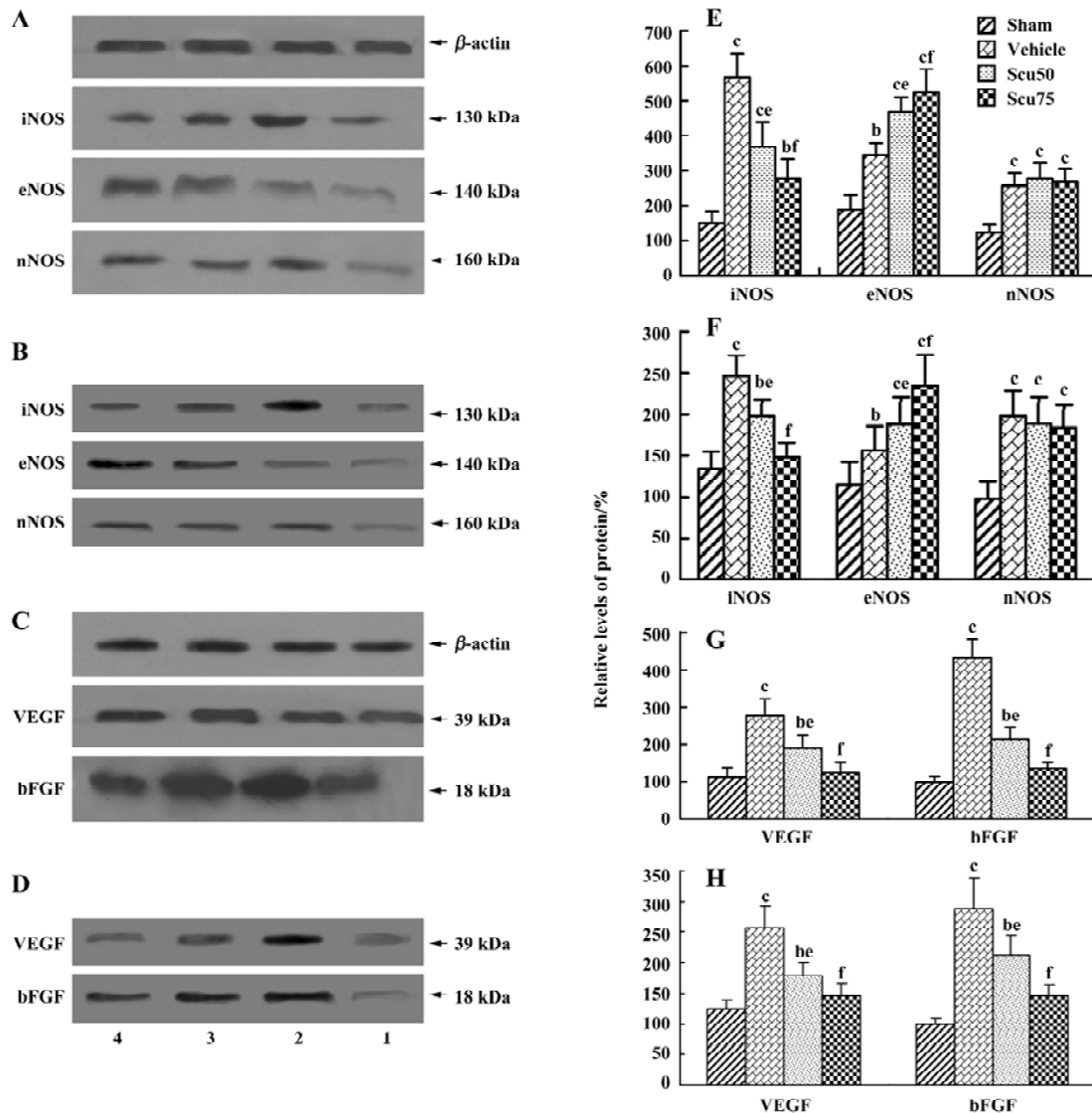


Figure 3. Western blot analysis of the effect of scutellarin on the expressions of NO synthase isoforms and angiogenic molecules both in the hippocampus and in the cortex after 2 h ischemia and 24 h reperfusion in rats. A, B: Protein expression of iNOS, eNOS and nNOS in the hippocampus (A) and in the cortex (B); C, D: protein expression of VEGF and bFGF in the hippocampus (C) and in the cortex (D). Upper panels: representative Western blot. Lane 1: sham; lane 2: vehicle; lane 3: 50 mg/kg scutellarin; lane 4: 75 mg/kg scutellarin; β -actin was used as an internal control. Lower panels (E, F, G, H): bar graph comparing the immunostained bands for each protein in the 4 groups (the relative abundance of the immunostaining) determined by the Image Quant program. E, F: Bar graphs for iNOS, eNOS and nNOS in the hippocampus (E) and in the cortex (F); G, H: bar graphs for VEGF and bFGF in hippocampus (G) and in the cortex (H). The levels of the 3 NOS isoforms and the angiogenic molecules are expressed as percentages of β -actin. $n=6$. Mean \pm SD. $n=6$. ^b $P<0.05$, ^c $P<0.01$ vs sham. ^e $P<0.05$, ^f $P<0.01$ vs vehicle (I/R+NS).

lar permeability factor that undergoes transcriptional and post-transcriptional induction by hypoxia; it couples hypoxia to angiogenesis in diverse tissues, including the brain^[23-26]. VEGF may also play an important role in the vascular response to cerebral ischemia, because ischemia stimulates VEGF expression in the brain, which promotes the formation of new cerebral blood vessels^[27,28]. It is thought that eNOS is involved in mediating the angiogenic molecules (VEGF and bFGF). Both factors induced eNOS expression, so eNOS

may be a downstream messenger in their angiogenic action. In the present study we also investigated the expression of the angiogenic molecules VEGF and bFGF in the hippocampus and in the cortex. In agreement with the results of previous reports^[29,30], our data showed that the expression of VEGF and bFGF in the hippocampus and cortex were upregulated in the vehicle-operated group after cerebral I/R. Scutellarin downregulated the expression of VEGF and bFGF. Further study is needed to shed light on the mechanisms involved in

the effect of scutellarin on the expression of eNOS (upregulation) and VEGF (downregulation) in I/R brain tissue.

In conclusion, scutellarin alleviated hippocampal neuronal dysfunction after cerebral I/R. This alleviation was accompanied by the effects of the molecular features of NOS isoforms and angiogenic molecules. These findings suggest that scutellarin exerts neuroprotective effects on brain injury induced by cerebral I/R, which allows a better understanding regarding the potential clinical therapeutic use of scutellarin.

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