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RESEARCH ARTICLE

Effect of ephrin-B2 on the expressions of angiopoietin-1 and -2 after focal cerebral ischemia/reperfusion

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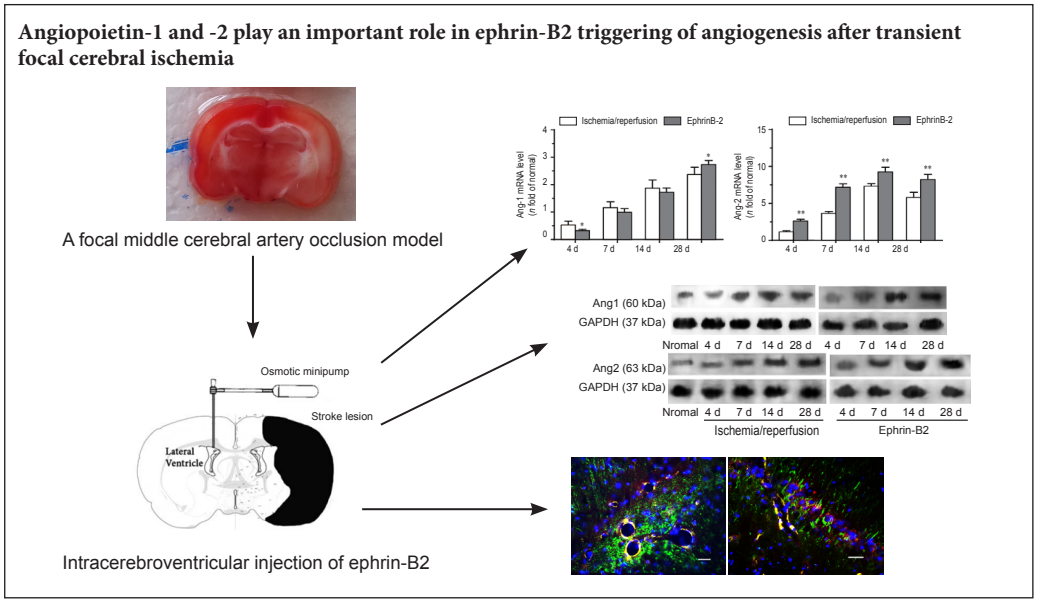
Graphical Abstract



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Abstract

Ephrin-B2 has been shown to participate in angiogenesis, but the underlying mechanisms involved remain unclear. In this study, a rat model of focal cerebral ischemia was prepared by focal middle cerebral artery occlusion, followed by 24-hour reperfusion. Then, ephrin-B2 protein was administered intracerebroventricularly for 3 consecutive days *via* a micro-osmotic pump. Western blot assay and quantitative real-time reverse transcription PCR demonstrated the expression levels of angiopoietin-1 (Ang-1) mRNA and protein in the penumbra cortex of the ephrin-B2 treated group were decreased at day 4 after reperfusion, and increased at day 28, while the expression levels of angiopoietin-2 (Ang-2) were highly up-regulated at all time points tested. Double immunofluorescent staining indicated that Ang-1 and Ang-2 were both expressed in vascular endothelial cells positive for CD31. These findings indicate that ephrin-B2 influences the expressions of Ang-1 and Ang-2 during angiogenesis following transient focal cerebral ischemia.

Key Words: *nerve regeneration; focal cerebral ischemia/reperfusion; ephrin-B2; angiogenesis; angiopoietin-1; angiopoietin-2; neuroprotection*

Introduction

Previous studies demonstrated that angiogenesis occurs (Krupinski et al., 1994) and plays a key role in the recovery of neurological function after cerebral infarction (Ergul et al., 2012). In addition, ephrin-B2 in endothelial cells promotes angiogenesis and endothelial cell proliferation through the signal transduction of Eph-B4 ligands, which is achieved by regulating the selective, detective and extensive functions of tip cells in angiogenic sprouting (Sawamiphak et al., 2010a; Sawamiphak et al., 2010b; Salvucci and Tosato, 2012). Our research indicated that ephrin-B2 was highly up-regulated in the ischemic penumbra after focal cerebral ischemia (Xiao et al., 2014a, b). Ephrin-B2 facilitated angiogenesis in the ischemic penumbra after cerebral ischemia in rats and

improved neurological function defects (Xiao et al., 2014a, b). However, the signaling pathway of ephrin-B2-regulated angiogenesis after cerebral ischemia has not been clarified. Angiopoietin protein families are the major effectors of angiogenesis, among which angiopoietin-1 (Ang-1) and -2 (Ang-2) are the most widely studied. In this study, we investigated the underlying mechanisms of ephrin-B2-regulated angiogenesis by administering ephrin-B2 protein intracerebroventricularly to mice *via* a micro-osmotic pump to examine the expression levels of Ang-1 and Ang-2 using western blot assay, PCR, and double immunofluorescence.

Materials and Methods

Preparation of cerebral ischemia/reperfusion rat models

Eighty-five adult male Sprague-Dawley rats of clean grade, weighing 280 ± 20 g, aged 8–10 weeks, were purchased from the Experimental Animal Center of Central South University (license No. SCXK (Xiang) 2011-0003). All rats were housed at room temperature (25°C) on a 12-hour light/dark cycle and allowed free access to food and water. All attempts were made to minimize the pain and distress of the experimental animals. The experimental protocol was in accordance with guidelines of Central South University and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23) and was approved by the Institutional Animal Care and Use Committee of Central South University (Approval No. 2012-11).

Eighty-five rats were randomly divided into three groups: five rats in a normal group underwent no intervention; 40 rats in an ischemia/reperfusion group were narcotized to prepare the middle cerebral artery occlusion (MCAO) model (Longa et al., 1989); and 40 rats in an ephrin-B2-Fc treated group were administered ephrin-B2-Fc followed by MCAO. According to the sampling time, rats in the ischemia/reperfusion and ephrinB2-Fc treated groups were randomly divided into day 4, 7, 14 and 28 subgroups (n = 10 per group).

Cerebral ischemia/reperfusion models were prepared as previously described (Longa et al., 1989). Sutures were performed with a nylon fishing line of 0.26 m diameter, dipped into nail polish at the 5 mm head end, smoothed on the surface, dried, and marked at the 18–20 mm end. The right middle cerebral artery of all the rats was occluded with the line plugged into the 18–20 mm end. When the iris color of the rat right eye turned a lighter color, this was recorded as the start of the ischemia. Two hours later, the suture was removed to achieve reperfusion. When the animal recovered from anesthesia, scoring was conducted using the Longa rating method (Longa et al., 1989) to determine whether the model was successful. A score of 1–3 indicated successful surgery. The room temperature was maintained at 20–30°C during surgery.

Intracerebroventricular injection of ephrin-B2-Fc *via* micro-osmotic pumps

At 24 hours after reperfusion, 10% chloral hydrate (0.35 mL/100 g) was used to anesthetize the rats and micro-osmotic pumps (Durect, Cupertino, CA, USA) were implanted subcutaneously into the rats. The head of the rat was stabilized by a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA), punctured at the lateral ventricle, with the anterior fontanelle as the base point of puncture (Kim et al., 2013), anchored at 2 mm to the left side of the midline, 2.6 mm behind the anterior fontanelle and 2 mm in depth. A needle was left *in situ* for 5 minutes to observe whether the puncture site was bleeding. Then micro-osmotic pumps were inserted at a 2 mm depth by intraventricular administration. Ten micrograms (concentration 100 μg/mL) of recombinant murine ephrin-B2-Fc chimera (R&D Systems, Minneapolis, MN, USA) was diluted with 0.9% sodium chloride and added to an osmotic pump (Durect), which was then injected into the lateral ventricle at a speed of 1 μL/hour for 3 days.

Double immunofluorescence staining

Five rats from each group were chosen at each time point. Normal saline and 4% paraformaldehyde were perfused through the heart to stabilize tissues, which were cut into frozen slices (40-μm thick) through the coronal plane of the caudate putamen. Their membranes were ruptured with 0.3% Triton and the sections were sealed with normal animal serum. Drops of primary antibody were added after the slices were rinsed. Primary antibody concentrations: anti-CD31 mouse monoclonal antibody (1:500, incubated for 48 hours at 4°C; Abcam, Cambridge, MA, USA), anti-Ang-l rabbit polyclonal antibody (1:100, incubated for 48 hours at 4°C; Abcam), and anti-Ang-2 rabbit polyclonal antibody (1:500, incubated for 48 hours at 4°C; Abcam). Appropriate fluorescent secondary antibodies were added after rinsing with PBS: goat anti-mouse IgG-Cy3 (1:50, incubated for 2 hours at 20°C; Boster, Wuhan, China) and goat anti-rabbit IgG-FITC (1:50, incubated for 2 hours at 20°C; Boster). The nucleus was marked after 5-minute 4′,6-diamidino-2-phenylindole (DAPI) staining. Slices were observed and photographed under a BX50 fluorescence microscope (Olympus Co., Tokyo, Japan). A confocal microscope (Zess, Oberkochen, Germany) was used to observe the stained slices and scanned images.

Western blot assay

Five rats from each group were selected and euthanized by cutting the brain quickly after intraperitoneal anesthesia with 10% chloral hydrate. About 100 mg of cerebral cortex in ischemic penumbra (7–11 mm after the frontal pole in the frontal plane) was separated from the brain on ice, proteolytic cleavage was conducted, and the brain tissues were grinded in an ice bath. The supernatant was separated using a centrifuge and protein concentrations were determined using the BCA method (Yang et al., 2012). Protein samples were transferred to a nitrocellulose membrane and sealed with skimmed milk after being separated by vertical electrophoresis. Subsequently, rabbit anti-Ang-l polyclonal antibody (1:500; Abcam), rabbit anti-Ang-2 polyclonal antibody (1:1,000; Abcam) and goat anti-rabbit IgG (1:2,000; Santa Cruz, Dallas, TX, USA) were added to the protein samples, the first two of which were incubated at 4°C overnight while the third was incubated at room temperature

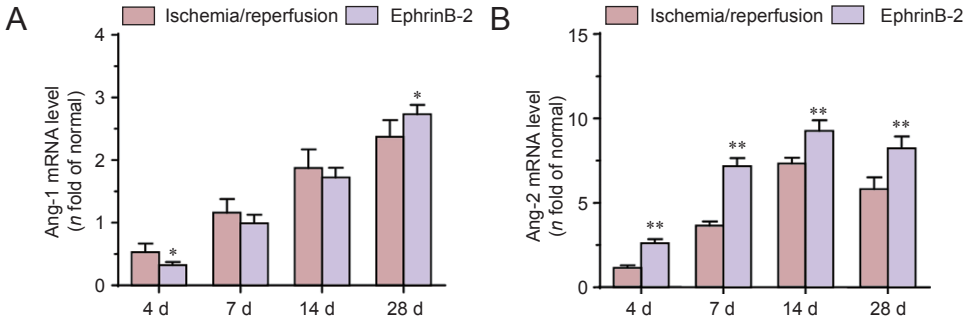

Figure 1 Effect of ephrin-B2 on Ang-1 and Ang-2 mRNA levels in the ischemic penumbra of rat cerebral cortex.

(A) Compared with the ischemia/reperfusion group, Ang-1 mRNA levels in the penumbra cortex in the ephrin-B2 treated group were decreased by day 4 and slightly up-regulated on day 28 (*P < 0.05, ischemia/reperfusion group). (B) Compared with the ischemia/reperfusion group, Ang-2 mRNA levels in the penumbra cortex in the ephrin-B2 treated group were slightly up-regulated after reperfusion in all the subgroups (**P < 0.01, *vs*. ischemia/reperfusion group). Data are expressed as the mean ± SD. Comparisons between groups were conducted by a two-tailed Student's *t*-test. Comparisons among groups were performed by analysis of variance. d: Days; Ang-1: angiopoietin-1; Ang-2: angiopoietin-2.

and exposed in an X-box after a luminous photosensitive agent (Biodragon, Beijing, China) was added. A gel imaging system (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) was utilized to analyze the scanned results and measure the mean absorbance values of protein bands. The ratio of absorbance values between GAPDH and the target protein band was the relative value of the target protein.

Fluorescence quantitative RT-PCR

About 100 mg of cerebral cortex in the ischemic penumbra was separated (the region of the penumbra was defined as a 7–11 mm range from the coronal plane to the frontal pole and the tissue region between the cerebral longitudinal fissure of the ischemic part and 1/3 of the upper portion of the lateral sulcus of the brain cortex, with a 1 mm inner longitudinal fissure removed). Total RNAs were extracted using TRIZOL one-step method (Zhou et al., 2012) and then reverse transcribed into cDNA. Fluorescence quantitative RT-PCR was used to detect the expression levels of Ang-1 and Ang-2 mRNA in brain tissues. Premier 5.0 (PREMIER Biosoft, Palo Alto, CA, USA) was used to design primers that were synthesized by Sangon Biotech (Shanghai, China). The upstream sequence of primers for the Ang-1 gene was 5′-CAC CGT GAG GAT GGA AGC CTA-3′, and the downstream sequence was 5′-TTC CCA AGC CAA TAT TCA CCA GA-3′, with a product length of 92 bp. The upstream sequence of primers for the Ang-2 gene was 5′-CAG TAG CAT CAG CCA ACC AGG A-3′, and the downstream sequence was 5′-GAC CAC ATG CGT CGA ACC AC-3′, with a product length of 116 bp. β-actin: Upstream 5′-CGT TGA CAT CCG TAA AGA C-3′, downstream 5′-TGG AAG GTG GAC AGT GAG-3′; with a product length of 201 bp. The reaction system included 2 μL cDNA. The volume of upstream and downstream primers was 0.4 μL each. The volume of DEPC-treated ultra-pure water and SYBR Green was 20 μL and 10 μL, respectively. The reaction was carried out in a LightCycler PCR thermal cycler (Roche Germany). Amplification conditions were as follows: preheated for 10 seconds at 95°C then 94°C for 45 seconds, 57°C for 1 minute, and 72°C for 1 minute for 30 circles, using the $2^{-\Delta\Delta Ct}$ (RQ) method (Schefe et al., 2006) to collect relative quantitative data.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD), and were compared by two-tailed Student's *t*-test and analysis of variance. P < 0.05 was considered statistically significance. All data were processed using the SPSS 13.0 statistical software package (SPSS, Chicago, IL, USA). Diagramming was completed by using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Effect of ephrin-B2 on Ang-1 and Ang-2 mRNA expression levels in the ischemic penumbra of rat cerebral cortex

Fluorescence quantitative RT-PCR indicated that Ang-1 mRNA levels in the ischemia/reperfusion group were decreased at 4 days after ischemia/reperfusion, started to increase at 7 days and peaked at 28 days compared with the normal group. Compared with the ischemia/reperfusion group, Ang-1 mRNA levels in the ephrin-B2 treated group were decreased at 4 days, and increased slightly at 28 days (P < 0.05; **Figure 1A**).

The Ang-2 mRNA levels in the ischemia/reperfusion and ephrin-B2 treated groups began to increase from day 4, reached a peak at 14 days, and were slightly reduced at 28 days after ischemia and reperfusion. The Ang-2 mRNA levels in the ephrin-B2 treated group were significantly higher than in the ischemia/reperfusion group (P < 0.01; **Figure 1B**).

Effect of ephrin-B2 on Ang-1 and Ang-2 protein levels in the ischemic penumbra of rat cerebral cortex

Western blot assay demonstrated that Ang-1 protein levels of the ischemia/reperfusion and ephrin-B2 treated groups were lower than those in the normal group after ischemia and reperfusion at 4 days, started to increase at 7 days, and peaked at 28 days. Compared with the ischemia/reperfusion group, the Ang-1 protein levels in the ephrin-B2 treated group was reduced at 4–14 days (P < 0.05 or P < 0.01)

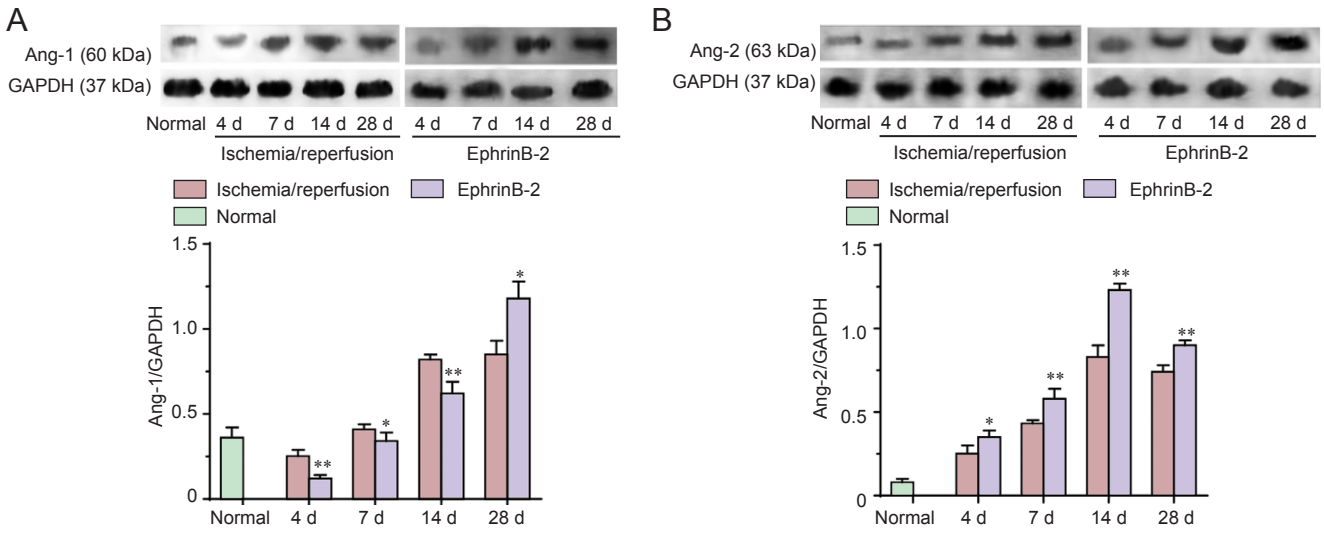

Figure 2 Effect of ephrin-B2 on the Ang-1 and Ang-2 protein levels in the ischemic penumbra of rat cerebral cortex.
(A) Compared with the ischemia/reperfusion group, Ang-1 protein levels in the penumbra cortex of the ephrin-B2 treated group were decreased at 4–14 days (*P < 0.05, **P < 0.01), and up-regulated on day 28 (*P < 0.05, *vs.* ischemia/reperfusion group). (B) Compared with the ischemia/reperfusion group, Ang-2 protein levels in the penumbra cortex of the ephrin-B2 treated group were markedly up-regulated after reperfusion in all the subgroups (*P < 0.05, **P < 0.01, *vs.* ischemia/reperfusion group). The relative expression of the target protein was expressed as the ratio of absorbance values between the target protein and GAPDH. Data are expressed as the mean ± SD. Comparison between groups was conducted by a two-tailed Student's *t*-test. Comparison among groups was performed by analysis of variance test. d: Days; Ang-1: angiopoietin-1; Ang-2: angiopoietin-2.

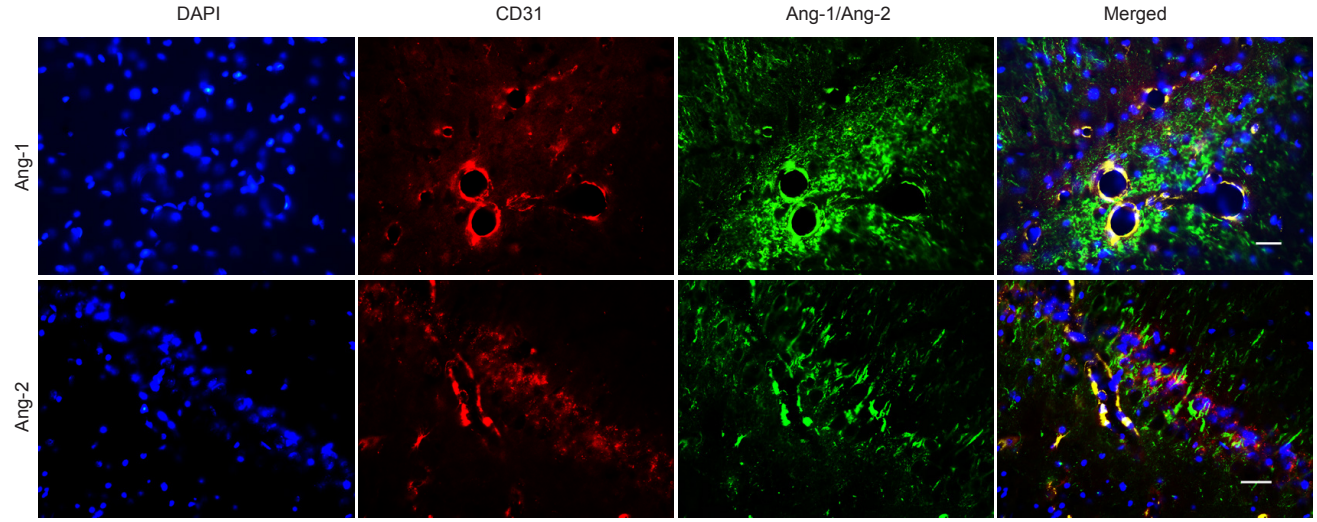

Figure 3 Effect of ephrin-B2 on Ang-1 and Ang-2 distribution in the ischemic penumbra of rat cerebral cortex.
Ang-1 (upper)/Ang-2 (lower) (green) colocalized with the blood vessel marker CD31 (red) in the ischemic border zone. DAPI = blue. Scale bars: 25 μm. Ang-1: Angiopoietin-1; Ang-2: angiopoietin-2.; DAPI: 4′,6-diamidino-2-phenylindole.

and significantly increased by day 28 (P < 0.01; **Figure 2A**). The Ang-2 protein levels in the ischemia/reperfusion and ephrin-B2 treated groups began to increase from day 4 after reperfusion, reached a peak at 14 days, and then decreased. In contrast to the ischemia/reperfusion group, a significant increase in Ang-2 protein levels was observed in the ephrin-B2 treated group (P < 0.05 or P < 0.01; **Figure 2B**).

Effect of Ephrin-B2 on the Ang-1 and Ang-2 distribution in the ischemic penumbra of rat cerebral cortex

Double immunofluorescence showed that Ang-1 and Ang-2 were mainly expressed in $CD31^+$ endothelial cells and perivascular cells in the ischemic border zone (**Figure 3**).

Discussion

Ischemia and hypoxia caused by cerebral ischemia are major factors that cause neural degeneration and necrosis, eventually leading to neurologic impairment (Weaver and Liu, 2015). Previous studies have shown that angiogenesis occurs in brain tissues after cerebral infarction (Krupinski et al., 1994). Angiogenesis can improve the blood supply to the brain, promote neural repair and assist in removing

necrotic tissues (Ergul et al., 2012; Qin et al., 2014), which is important for the prognosis of patients with cerebral ischemia. Ephrins/Eph are one of the ligand receptor systems of the tyrosine kinase receptor, which is a key factor in angiogenesis and vasculogenesis (Jeltsch et al., 2013). Ephrin-B2 participates in angiogenesis and the regulation of axonal growth simultaneously (Brantley-Sieders and Chen, 2004). Studies have demonstrated that a lack of Eph-B4 or Ephrin-B2 in rat embryos caused serious cardiovascular malformations (Wang et al., 2010). Intraperitoneal injection of ephrin- B2 protein promoted angiogenesis in ischemic tissues (Månsson-Broberg et al., 2008). Our previous research showed a marked increase in the expression of ephrin-B2 in the ischemic border area after focal cerebral ischemia/reperfusion (Xiao et al., 2014a, b). Continuous injection of recombinant murine ephrin-B2-Fc chimera into the lateral ventricle *via* a micro-osmotic pump promoted angiogenesis and neurological repair (Xiao et al., 2014a, b) .

Ang is another angiogenesis regulatory factor family identified after the discovery of vascular endothelial growth factor (VEGF), among which Ang-1 and Ang-2 are most widely studied (Davis et al., 1996; Valenzuela et al., 1999). Tie-2 is the common receptor of Ang-1 and Ang-2, but Tie-2 has a different biological effect from Ang-1 and Ang-2. Ang-1 binds and phosphorylates Tie-2, which prevents perithelial cells from breaking away from endothelial cells and enables vessels to maintain a stable state (Stoeltzing et al., 2003). Ang-2 binds to the endothelial Tie-2 receptor but does not phosphorylate the Tie-2 receptor, which competitively blocks the effects of Ang-1, reduces vessel stability, and breaks the vascular structures. This process releases the constraints of perivascular cells and extracellular matrix on endothelial cells, which allows endothelial cells to extravasate through the existing blood vessels and enhance the capacity of endothelial cell proliferation and angiogenesis of VEGF (Maisonpierre et al., 1997). Ang-1 is widely expressed in vascular pericytes and vascular smooth muscle cells (Davis et al., 1996). Hypoxia and inflammatory cytokine stimulation increased the expression of Ang-2 on endothelial cells (Krikun et al., 2000; Yuan et al., 2000), where Ang-2 is mainly expressed (Park et al., 2003). Numerous studies have shown that cerebral ischemia has a marked effect on the expression levels of Ang-1 and Ang-2; however, the changes in Ang-1 and Ang-2 expression are different. After focal cerebral ischemia in rats, Ang-2 mRNA expression was increased after 12 hours of ischemia, reached a peak after 24 hours, declined after 72 hours and increased again 14 days later (Lin et al., 2000). *In situ* hybridization showed that Ang-2 mRNA was expressed at the first layer of the infarcted cortex that is an angiogenesis-active region. The expression level of Ang-1 mRNA increased gradually after 7 days and reached a peak after 14 days.

Our study found that both Ang-1 and Ang-2 were expressed in brain tissues in normal rats, but Ang-1 levels changed more significantly than Ang-2, which was associated with the role of Ang-1 in maintaining the stability of existing blood vessels in the normal physiological state. Our research also showed that Ang-1 mRNA expression and protein levels in the ischemic border zone were lower than those in the normal group after ischemia and reperfusion at 4 days, started to increase at 7 days, and reached a peak at 28 days. Ang-2 mRNA expression and protein levels reached a peak at day 14 and then slightly reduced at 28 days, followed by an increase at 4 days after ischemia and reperfusion. Immunofluorescence staining demonstrated that both Ang-1 and Ang-2 were expressed in vessels of the ischemic border. Our research, taken together with previous studies, suggest that the expression of Ang-2 was enhanced at the early stage of cerebral ischemia, while the expression of Ang-1 was decreased, which reduced the stability of vessels and vascular structures. This change also released the constraint of perivascular cells and extracellular matrix on endothelial cells allowing endothelial cells to extravasate from the blood vessels to promote angiogenesis. At the subacute stage of cerebral ischemia, the down-regulated Ang-2 and up-regulated Ang-1 helped prevent the perithelial cells from detaching from the endothelial cells and accelerate maturation and neurovascular stabilization.

Our study also found that compared with the subgroups of the ischemia/reperfusion group, Ang-1 mRNA expression and protein levels of the ephrin-B2 treated group were decreased at 4 days after ischemia/reperfusion, and increased at 28 days, while Ang-2 mRNA expression and protein levels increased remarkably. In view of the biological effects of Ang-1 and Ang-2, we believe that at the early stage of ischemia/reperfusion, ephrin-B2 inhibits the expression of Ang-1 and promotes the expression of Ang-2, which facilitates the sprouting growth of endothelial cells to form new blood vessels. At the subacute stage of ischemia, ephrin-B2 up-regulates the expressions of Ang-1 and Ang-2, which is beneficial for endothelial cell proliferation and neurovascular maturation.

Author contributions: *HX and WPG conceived and designed this study. HX and JQW performed the experiment and wrote the paper. QH and QQD were responsible for data collection, analysis and interpretation, and participated in the writing of the paper. JQW and QQD provided assistance in technical application or material use. HX and WPG analyzed the experimental data. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
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References

Brantley-Sieders DM, Chen J (2004) Eph receptor tyrosine kinases in angiogenesis: from development to disease. Angiogenesis 7:17-28.
Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161-1169.
Ergul A, Alhusban A, Fagan SC (2012) Angiogenesis: a harmonized target for recovery after stroke. Stroke 43:2270-2274.
Jeltsch M, Leppänen VM, Saharinen P, Alitalo K (2013) Receptor tyrosine kinase-mediated angiogenesis. Cold Spring Harb Perspect Biol doi: 10.1101/cshperspect.a009183.

Kim SH, Yu HS, Park HG, Ha K, Kim YS, Shin SY, Ahn YM (2013) Intracerebroventricular administration of ouabain, a Na/K-ATPase inhibitor, activates mTOR signal pathways and protein translation in the rat frontal cortex. Prog Neuropsychopharmacol Biol Psychiatry 45:73-82.

Krikun G, Schatz F, Finlay T, Kadner S, Mesia A, Gerrets R, Lockwood CJ (2000) Expression of angiopoietin-2 by human endometrial endothelial cells: regulation by hypoxia and inflammation. Biochem Biophys Res Commun 275:159-163.

Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1994) Role of angiogenesis in patients with cerebral ischemic stroke. Stroke 25:1794-1798.

Lin TN, Wang CK, Cheung WM, Hsu CY (2000) Induction of angiopoietin and Tie receptor mRNA expression after cerebral ischemia-reperfusion. J Cereb Blood Flow Metab 20:387-395.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84-91.

Månsson-Broberg A, Siddiqui AJ, Genander M, Grinnemo KH, Hao X, Andersson AB, Wärdell E, Sylvén C, Corbascio M (2008) Modulation of ephrinB2 leads to increased angiogenesis in ischemic myocardium and endothelial cell proliferation. Biochem Biophys Res Commun 373:355-359.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55-60.

Park YS, Kim NH, Jo I (2003) Hypoxia and vascular endothelial growth factor acutely up-regulate angiopoietin-1 and Tie2 mRNA in bovine retinal pericytes. Microvasc Res 65:125-131.

Qin W, Yang L, Guo H, Xiang N, Hu W (2014) Pro-urokinase promotes angiogenesis but does not reduce neuronal apoptosis in infarcted cerebral tissue. Neural Regen Res 9:502-503.

Salvucci O, Tosato G (2012) Essential roles of EphB receptors and EphrinB ligands in endothelial cell function and angiogenesis. Adv Cancer Res 114:21-57.

Sawamiphak S, Ritter M, Acker-Palmer A (2010a) Preparation of retinal explant cultures to study ex vivo tip endothelial cell responses. Nat Protocols 5:1659-1665.

Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A (2010b) Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature 465:487-491.

Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. J Mol Med (Berl) 84:901-910.

Stoeltzing O, Ahmad SA, Liu W, McCarty MF, Wey JS, Parikh AA, Fan F, Reinmuth N, Kawaguchi M, Bucana CD, Ellis LM (2003) Angiopoietin-1 inhibits vascular permeability, angiogenesis, and growth of hepatic colon cancer tumors. Cancer Res 63:3370-3377.

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S, Yancopoulos GD (1999) Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci U S A 96:1904-1909.

Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, Barberis A, Benjamin LE, Makinen T, Nobes CD, Adams RH (2010) Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465:483-486.

Weaver J, Liu KJ (2015) Does normobaric hyperoxia increase oxidative stress in acute ischemic stroke? A critical review of the literature. Med Gas Res 5:11.

Xiao H, Gu W, Yang Q, Zeng X (2014a) Expression of Ephrin-B2 after focal cerebral ischemia in rats. Zhong Nan Da Xue Xue Bao Yi Xue Ban 39:452-457.

Xiao H, Gu WP, Hu J, Wang Z (2014b) Ephrin-B2 enhances expression of VEGF and angiogenesis after focal cerebral ischemia in rats. Xiandai Shengwu Yixue Jinzhan 14:5824-5828.

Yang AL, Zhou HJ, Lin Y, Luo JK, Cui HJ, Tang T, Yang QD (2012) Thrombin promotes the expression of thrombospondin-1 and -2 in a rat model of intracerebral hemorrhage. J Neurol Sci 323:141-146.

Yuan HT, Yang SP, Woolf AS (2000) Hypoxia up-regulates angiopoietin-2, a Tie-2 ligand, in mouse mesangial cells. Kidney Int 58:1912-1919.

Zhou HJ, Tang T, Cui HJ, Yang AL, Luo JK, Lin Y, Yang QD, Li XQ (2012) Thrombin-triggered angiogenesis in rat brains following experimental intracerebral hemorrhage. J Neurosurg 117:920-928.

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