Changes of hypoxia-inducible factor-1 signaling and the effect of cilostazol in chronic cerebral ischemia

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Research Highlights
(1) Hypoxia-inducible factor-1 under hypoxia is a hot topic in the field of neural regeneration research. Under hypoxia and ischemia/reperfusion, heme oxygenase-1 is upregulated by hypoxia-inducible factor-1. The available research mainly focuses on the role of hypoxia-inducible factor-1 and heme oxygenase-1 following acute cerebral ischemia and hypoxia, while very few studies have examined changes in the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway.
(2) This is the first report showing that the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway is activated and sustained following chronic cerebral ischemia.
(3) Hypoxia-inducible factor-1 and heme oxygenase-1 expression was downregulated by cilostazol in rats subjected to chronic cerebral ischemia. Our findings are the first to show that cilostazol protects against apoptosis in the frontal cortex of chronic cerebral ischemic rats. Cilostazol can provide protection against vascular cognitive impairment through its anti-apoptotic effect.

Abstract
Hypoxia-inducible factor-1 and its specific target gene heme oxygenase-1, are involved in acute cerebral ischemia. However, very few studies have examined in detail the changes in the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway in chronic cerebral ischemia. In this study, a rat model of chronic cerebral ischemia was established by permanent bilateral common carotid artery occlusion, and these rats were treated with intragastric cilostazol (30 mg/kg) for 9 weeks. Morris water maze results showed that cognitive impairment gradually worsened as the cerebral ischemia proceeded. Immunohistochemistry, semi-quantitative PCR and western blot analysis showed that hypoxia-inducible factor-1α and heme oxygenase-1 expression levels increased after chronic cerebral ischemia, with hypoxia-inducible factor-1α expression peaking at 3 weeks and heme oxygenase-1 expression peaking at 6 weeks. These results suggest that the elevated levels of hypoxia-inducible factor-1α may upregulate heme oxygenase-1 expression following chronic cerebral ischemia and that the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway is involved in the development of cognitive impairment induced by chronic cerebral ischemia. Cilostazol treatment alleviated the cognitive impairment in rats with chronic cerebral ischemia, decreased hypoxia-inducible factor-1α and heme oxygenase-1 expression levels, and reduced apoptosis in the frontal cortex. These findings demonstrate that cilostazol can protect against cognitive impairment induced by chronic cerebral ischemic injury through an anti-apoptotic mechanism.

Key Words
neural regeneration; chronic cerebral ischemia; cognitive impairment; hypoxia-inducible factor-1; heme oxygenase-1; cilostazol; apoptosis; grants-supported paper; neuroregeneration

INTRODUCTION

The transcriptional regulator hypoxia-inducible factor-1 has been the focus of much attention since it was first discovered in a study examining hypoxic induction of erythropoietin gene expression in 1992[1]. Hypoxia-inducible factor-1 is a critical component of the cellular and systemic response to hypoxia in mammals[2]. Several dozen target genes that are transactivated by hypoxia-inducible factor-1 have been identified[3-4], including erythropoietin, vascular endothelial growth factor, placental growth factor and heme oxygenase-1. These target genes are involved in hypoxic adaptation[5], inflammation[6], cell proliferation[7], angiogenesis and remodeling[8], erythropoiesis[9], iron transport[10], energy metabolism[11], apoptosis[12], tumor growth, and drug resistance[13]. Hypoxia-inducible factor-1 plays a pivotal role in the regulation of oxygen balance in cells. Ischemia and hypoxia induce hypoxia-inducible factor-1 by inhibiting its degradation. Hypoxia-inducible factor-1 is composed of α and β subunits, and the physiological activity of hypoxia-inducible factor-1 is mainly dependent on the activity and expression of the hypoxia-inducible factor-1α subunits[14]. Heme oxygenase-1 is a specific target gene of hypoxia-inducible factor-1[15]. Under hypoxia, ischemia, stress or other conditions, heme oxygenase-1 synthesis significantly increases in the brain[16-17]. Hypoxia-inducible factor-1 participates in the hypoxic response along with heme oxygenase-1, and induces heme oxygenase-1 gene expression under hypoxic conditions[18].

Chronic cerebral ischemia[19] refers to the pathological cerebral metabolic dysfunction and functional decline caused by long-term and chronic insufficiency of cerebral blood flow. It is considered a common pathological process in vascular dementia and Alzheimer’s disease. Changes in the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway in rats with cognitive impairment induced by chronic cerebral ischemia have been barely investigated. Cilostazol alleviates vascular cognitive impairment due to chronic cerebral ischemia and exerts a neuroprotective effect[20-22]. However, the mechanism of action of the drug remains elusive, and its link to the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway is unclear.

In this study, we employ the permanent bilateral common carotid artery occlusion model of cerebral ischemia in rats in an attempt to clarify the role of the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway in chronic cerebral ischemia-induced vascular cognitive impairment. We also examine the effect and mechanism of action of cilostazol on dementia in this model of chronic cerebral ischemia.

RESULTS

Quantitative analysis of experimental animals

The rats exhibited no apparent visual disturbance, difficulty with ingestion or marked motor impairment. All the rats included in this study were autopsied and no tumors or other pathological lesions were found. Rats with poor swimming ability or intellectual retardation were excluded with the Morris water maze test before grouping. Of the 110 rats in total, 30 formed part of the sham operated group, and 80 received permanent bilateral common carotid artery occlusion (2-vessel occlusion). Sixteen rats died because of ischemic seizure within 48 hours after 2-vessel occlusion. A total of 94 rats were included in the final analysis; 30 in the sham operated group, 46 in the cerebral ischemia group, and 18 in the cilostazol group.
analysis at 3, 6 and 9 weeks after operation. Ten animals were used for each time point for the sham operated group. For the cerebral ischemia group, fifteen animals each were used for the first two time points, while 16 were used for the final time point. The rats in the cilostazol group were intragastrically injected with cilostazol following cerebral ischemia and killed 9 weeks after operation.

**Learning and memory abilities of chronic cerebral ischemic rats**

Before permanent bilateral common carotid artery occlusion, all rats were subjected to place navigation test and spatial probe test using the Morris water maze. No differences in intelligence or swimming speed were found among the groups.

Place navigation test and spatial probe test at 3, 6 and 9 weeks after artery occlusion showed that the learning and memory abilities of rats declined with increasing duration of ischemia. In the place navigation test, the escape latency and swimming distance in the cerebral ischemia group were significantly longer compared with the sham operated group ($P < 0.05$). In the spatial probe test, the frequency of crossing the platform in the cerebral ischemia group was significantly lower than in the sham operated group ($P < 0.05$; Figure 1). These results indicate that rats in the cerebral ischemia group exhibited poor behavior performance over the course of behavioral testing.

Cilostazol treatment for 9 weeks reduced the escape latency and swimming distance, and significantly increased the frequency of crossing the platform ($P < 0.05$). These findings indicate that cilostazol alleviated the cognitive impairment in rats with chronic cerebral ischemia (Figure 1).

**Hypoxia-inducible factor-1α and heme oxygenase-1 immunoreactive cells in the frontal cortex of chronic cerebral ischemic rats detected with immunohistochemistry**

In the frontal cortex, immunoreactivity for hypoxia-inducible factor-1α was mainly localized to the nucleus, while immunoreactivity for heme oxygenase-1 was localized to the cytoplasm. In the sham operated group, the distribution and number of neurons were normal, and the neurons had round and clear nuclei. Immunolabeled cells were rare in the sham operated group. In the cerebral ischemia group, hypoxia-inducible factor-1α and heme oxygenase-1 immunolabeling was observed in the ischemic frontal cortex, and the signal intensities were significantly increased compared with the sham operated group ($P < 0.05$). These cells with varying intensities of immunolabeling, with a polygonal shape, were greater in number in the ischemic brain than in the corresponding regions of sham operated rats. Long protruding neurites were visible in some of the immunolabeled cells. The most robust immunolabeling for hypoxia-inducible factor-1α and heme oxygenase-1 was found at 3 and 6 weeks after ischemia, respectively (Figure 2).

**Figure 1** Behavioral performance of chronic cerebral ischemic rats and effects of cilostazol treatment. 

(A) Escape latency in the place navigation test. 
(B) Swimming distance in the place navigation test. 
(C) Frequency of crossing the platform in the spatial probe test. $^aP < 0.05$, vs. sham operated group (Sham); $^bP < 0.05$, vs. cerebral ischemia group (Ischemia).

There were 10 rats in the sham operated group at each time point, and 15 rats at 3 weeks, 15 rats at 6 weeks and 16 rats at 9 weeks in the cerebral ischemia group, as well as 18 rats at 9 weeks in the cilostazol group. Data are expressed as mean ± SD and analyzed using one-way analysis of variance.

**The mRNA and protein expression levels of hypoxia-inducible factor-1α and heme oxygenase-1 in the frontal cortex of chronic cerebral ischemic rats**

Semi-quantitative reverse-transcription (RT)-PCR assay detected a hypoxia-inducible factor-1α PCR product of

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Expression of hypoxia-inducible factor-1α mRNA was very weak in the sham operated group. In the cerebral ischemia group, the hypoxia-inducible factor-1α band was visible at each time point, and reached a peak at 3 weeks.

Hypoxia-inducible factor-1α expression then declined progressively, but remained above the sham operated group (P < 0.05). The absorbance ratio (to β-actin) was used as an indicator of the mRNA expression level of target genes. Heme oxygenase-1 was weakly expressed in the cerebral ischemia groups, but this expression level was higher than in the sham operated group (P < 0.05). The expression rose at 3 weeks, peaked at 6 weeks, and then declined at 9 weeks (Figure 3). Western blot analysis showed that hypoxia-inducible factor-1α and heme oxygenase-1 protein levels paralleled the mRNA levels determined with RT-PCR assay (Figure 4).

RT-PCR and western blot analysis demonstrated that the levels of hypoxia-inducible factor-1α and heme oxygenase-1 were downregulated by cilostazol treatment. There were statistically significant differences in mRNA and protein levels of hypoxia-inducible factor-1α and heme oxygenase-1 between the cilostazol and cerebral ischemia groups at 9 weeks of chronic cerebral ischemia (P <
Anti-apoptotic effect of cilostazol in chronic cerebral ischemic rats

Flow cytometric analysis showed that cilostazol significantly reduced cellular apoptosis in the frontal cortex of rats with chronic cerebral ischemia at 9 weeks (subdiploid peak in Figure 5). The percentage of apoptotic cells in the frontal cortex of rats in the cerebral ischemia group was higher than in the sham operated group, and was reduced significantly in the cilostazol group compared with the cerebral ischemia group ($P < 0.05$).

**DISCUSSION**

Adaptation to hypoxia is a necessity for the great majority of living organisms, whether under physiological or pathological conditions. Hypoxia is the major pathophysiological component of cerebral ischemic disease. Hypoxia-inducible factor-1 has received increasing and sustained attention for its key role in ischemia and hypoxia. Under hypoxia, hypoxia-inducible factor-1 accumulates and binds to the regulatory regions of its downstream target genes, inducing their expression$^{[23-24]}$. As revealed by the functions of its target genes, hypoxia-inducible factor-1 is an essential transcription factor controlled by changes in oxygen concentration$^{[25]}$, and is a key mediator of physiological and pathophysiological responses to hypoxia. Hypoxia-inducible factor-1α, the biologically active component of hypoxia-inducible factor-1$^{[25]}$, rapidly increases in concentration under hypoxia and is rapidly degraded when oxygen supply recovers. Growing evidence shows that hypoxia-inducible factor-1 is induced by cerebral ischemia. Using a rat model of permanent middle cerebral artery occlusion, Bergeron et al$^{[26]}$ were the first to find that mRNA encoding hypoxia-inducible factor-1α was upregulated in the peri-

![Figure 4](image4.png)

**Figure 4** Protein expression levels of hypoxia-inducible factor-1α (HIF-1α) and heme oxygenase-1 (HO-1) in the frontal cortex of chronic cerebral ischemic rats.

There were 10 rats in the sham operated group (Sham) at each time point, and 15 rats at 3 weeks, 15 rats at 6 weeks and 16 rats at 9 weeks in the cerebral ischemia group (Ischemia), as well as 18 rats at 9 weeks in the cilostazol group.

Data are presented as absorbance ratio of target protein to β-actin protein, expressed as mean ± SD and analyzed using one-way analysis of variance. $^aP < 0.01$, vs. sham operated group; $^bP < 0.05$, vs. ischemia group.

![Figure 5](image5.png)

**Figure 5** Anti-apoptotic effect of cilostazol in the frontal cortex of chronic cerebral ischemic rats.

Nine weeks after chronic cerebral ischemia, 10 rats in the sham operated group (Sham), 16 rats in the cerebral ischemia group (Ischemia), and 18 rats in the cilostazol group were killed for flow cytometric analysis. The number refers to the percentage of apoptotic cells.

Data are expressed as mean ± SD, and analyzed using one-way analysis of variance. $^aP < 0.05$, vs. sham operated group; $^bP < 0.05$, vs. cerebral ischemia group.
infarct penumbra 7.5 hours after focal ischemia in the brain. Their findings suggest that hypoxia-inducible factor-1α might contribute to the neuroprotection mediated by hypoxic pre-conditioning in the newborn brain. Furthermore, hypoxia-inducible factor-1α rapidly accumulates during the onset of hypoxia and remains at elevated levels for 14 days in rats subjected to chronic hypoxia[27]. Neurons, astrocytes, ependymal cells, and possibly endothelial cells express hypoxia-inducible factor-1α. Tipoe et al[28] showed that hypoxia-inducible factor-1α expression is upregulated in vivo by chronic hypoxia. A broad spectrum of stimuli can induce heme oxygenase-1 expression, including extreme oxygen environments and ischemia/reperfusion injury[29-30]. In the present study, western blot analysis and immunocytochemistry demonstrated that hypoxia-inducible factor-1α protein significantly accumulated in the rat brain during chronic cerebral ischemia, indicating ischemia-inducible expression of hypoxia-inducible factor-1α in the brain. The accumulation of hypoxia-inducible factor-1α induced by hypoxia mainly arises from inhibition of the intracellular oxygen-dependent ubiquitin-proteasome degradation pathway[31]. However, the RT-PCR assay in our study showed that hypoxia-inducible factor-1α mRNA levels also increase in the ischemic rat brain, suggesting that certain growth factors and cytokines may upregulate hypoxia-inducible factor-1α gene expression under ischemic conditions; phosphatidylinositol 3-kinase and signal transducer and activator of transcription 3 are potential candidates[32-33]. We found that the patterns of heme oxygenase-1 and hypoxia-inducible factor-1α expression in the brain of 2-vessel occluded rats paralleled each other, indicating that both factors are nearly simultaneously activated by chronic cerebral ischemia. The observation that the peak of heme oxygenase-1 expression was later than that of hypoxia-inducible factor-1α supports the hypothesis that enhanced expression of hypoxia-inducible factor-1α contributes to heme oxygenase-1 upregulation. Hypoxia-inducible factor-1α protein levels reached a peak at 3 weeks in rats subjected to 2-vessel occlusion, and progressively declined during prolonged ischemia, but remained significantly elevated for at least 9 weeks compared with the sham operated group. These findings suggest that acute ischemic responses gradually diminish over time because of compensatory and adaptive mechanisms of cells, which lead to recovery[34-35]. In summary, the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway participates in the pathology of chronic cerebral ischemia in 2-vessel occluded rats.

It has been well documented that chronic cerebral ischemia is associated with cognitive decline in aging and Alzheimer’s disease[34]. There was a significant difference in behavioral performance between the ischemia and sham operated groups. In the ischemia group, learning and memory ability declined with increasing duration of ischemia, as previously described[36]. Results from the spatial probe test were similar to those from the place navigation test. The behavioral performance together with the mRNA and protein assay results suggest that the hypoxia-inducible factor-1 and heme oxygenase-1 signaling pathway is involved in cognitive impairment induced by chronic cerebral ischemia in rats.

The neuroprotective effects of cilostazol in acute cerebral ischemia are well documented[37-38]. Zhang et al[39] reported that cilostazol has protective effects in chronic cerebral injury. Thirty-five days after focal cerebral ischemia in mice, it significantly alleviated the neurological deficit, and increased the density of surviving neurons in the border of the ischemic region. Torigoe et al[40] examined the safety and efficacy of cilostazol in 24 patients subjected to chronic cerebral circulatory insufficiency, and found that the total improvement rate was 52.2%, which correlated positively with improvement in cerebral blood flow. In addition, cilostazol prevents Aβ25-35-induced short-term and long-term memory impairment in the Y-maze and the step-down type passive avoidance tests, respectively[41]. Cilostazol significantly improves spatial learning and memory in Aβ-injected mice by significantly decreasing ApoE-mediated Aβ aggregation[42]. Furthermore, concurrent treatment with cilostazol and donepezil improves spatial learning memory in rats subjected to chronic cerebral hypoperfusion[43]. Zhao et al[44] demonstrated that cilostazol administration might improve cognitive function in mice by increasing the hippocampal production of insulin-like growth factor-1. Cilostazol significantly improved spatial learning memory by reducing white matter damage, and exerted an anti-apoptotic effect in the corpus callosum by upregulating Bcl-2 expression in a rat model of chronic cerebral hypoperfusion[45]. Other studies have also shown that cilostazol has an anti-apoptotic effect in cerebral ischemia[46-47]. Bennett[48] and Tomimoto[49] both examined apoptotic neuronal cell death elicited by chronic cerebral hypoperfusion. In the present study, we show that cilostazol significantly reduces apoptosis in the frontal cortex 9 weeks after artery occlusion, accompanied by improved spatial memory. These findings indicate that cilostazol protects against cognitive dysfunction, at least in part, by exerting an anti-apoptotic effect in brain regions associated with learning and memory.

Furthermore, our findings showed that the levels of hy-
hypoxia-inducible factor-1α and heme oxygenase-1 were downregulated by cilostazol in rats subjected to 2-vessel occlusion for 9 weeks. This reduction in the chronic cerebral ischemia-mediated increase in hypoxia-inducible factor-1α and heme oxygenase-1 levels can have two alternative underlying causes. First, cilostazol may have reduced the severity of ischemia and hypoxia, which is the trigger for hypoxia-inducible factor-1α accumulation, thereby reducing hypoxia-inducible factor-1α accumulation. Alternatively, the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway may have been directly suppressed by cilostazol. Although hypoxia-inducible factor-1 and heme oxygenase-1 can protect against a variety of stressors, including cerebral ischemia, there is evidence suggesting that these factors have both positive and negative effects in ischemic and hypoxic conditions. The effect of hypoxia-inducible factor-1 on neural tissue injuries is debatable. In cultured cortical neurons, hypoxia-inducible factor-1α promotes cell death in the context of cerebral ischemia. Hypoxia-inducible factor-1α can promote hypoxia-induced apoptosis via two mechanisms; one is by stabilizing p53, the other is by inducing the proapoptotic protein BNIP3. The BNIP3 promoter contains an HRE. Thus, hypoxia-inducible factor-1 can induce the expression of this gene. NOS2, the product of another known hypoxia-inducible factor-1 target gene, also has a pro-apoptotic role during cerebral ischemia. In an ischemic stroke model, the tissue damage in hypoxia-inducible factor-1α-null adult mice was less than in control mice, and the expression levels of some pro-apoptotic genes were significantly downregulated. Heme oxygenase-1-induced CO can regulate the immune response and suppress apoptosis through cGMP, but prolonged upregulation of CO release increases oxidative stress injury. Heme oxygenase-1 may lead to cortical demyelination, which is an important process during development and in the progression of neurodegenerative diseases, such as Alzheimer's disease. Thus, the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway has opposing effects in chronic cerebral ischemia. Cilostazol might reduce activation of this pathway, thereby exerting an anti-dementia effect in the 2-vessel occluded rat model. The mechanism by which cilostazol regulates the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway remains to be determined in detail and deserves further study.

In conclusion, the hypoxia-inducible factor-1/heme oxygenase-1 signaling pathway contributes to the development and progression of cognitive impairment induced by chronic cerebral ischemia. This pathway is downregulated by cilostazol. Cilostazol has a neuroprotective effect in chronic cerebral ischemia through an anti-apoptotic mechanism in the brain in vivo. Our results provide experimental support for the pharmacological application of cilostazol for the treatment of vascular cognitive impairment.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
The experiment was performed at the School of Pharmaceutical Sciences, and the Prostate Disease Prevention Research Center, Jilin University, China from September 2007 to September 2009.

**Materials**

**Animals**
Adult male Wistar rats, weighing 250–280 g, aged 2 months, were purchased from the Experimental Animal Center of Jilin University, China (certificate No. SYXK (Ji) 2008-0010/0011). All the rats were housed in clean polypropylene cages with 12-hour light/dark cycles for at least 1 week before the experiments. The rooms were equipped with air conditioning equipment to maintain the temperature at 23 ± 2°C and the humidity at 50 ± 5%. Food and water were provided ad libitum.

**Drugs**
Cilostazol was provided by China Otsuka Pharmaceutical Co., Ltd., China (approval No. H10960014; 50 mg/tablet). Cilostazol was dissolved in 25% dimethyl sulfoxide solution for use.

**Methods**

**Establishment of chronic cerebral ischemia model and drug administration**
Chronic cerebral ischemia was induced by permanent bilateral common carotid artery occlusion (2-vessel occlusion) Rectal temperature was maintained at 37–38°C using a heated blanket during the whole surgical procedure. While the bilateral carotid arteries were occluded, the rat tail was cut to antagonize the sudden increase in blood pressure, which can lead to heart failure and death. After surgery, penicillin (20 million units/kg body weight) was intramuscularly injected daily for 3 consecutive days. Cilostazol solution was intragastrically administered at a dose of 30 mg/kg into rats in the cilostazol group, once daily for 9 weeks (the first time
less than 24 hours after operation). 25% dimethyl sulfoxide solution was used as vehicle in the cerebral ischemia group. The rats in the sham operated group were treated similarly to the operated ones, except that the common carotid arteries were not occluded.

**Assessment of learning and memory abilities**

Place navigation test and spatial probe test were performed using the Morris water maze, which is composed of a circular pool, automatic camera and computer analysis system (Olympus, Tokyo, Japan). Before the test, the circular pool wall was marked with four equally-spaced entry points. The circular pool was thereby divided into four quadrants. A platform (diameter 11 cm) was placed at the center of one quadrant and immersed 1 cm under the water surface during acquisition trials. Milk powder was added into the pool water to prevent rats from seeing the platform. The water temperature (26 ± 1°C), light intensity, external cues in the room, and water opacity were rigorously controlled and kept unchanged throughout the behavioral test. The rat’s head was stained before the test to allow the camera to track and record the animal’s movement.

The main procedures in the place navigation test were as follows: The rats were released into the water facing the pool wall at one of the four entry points. The time for the animal to reach the hidden platform was recorded as escape latency, and the distance traveled in finding the platform was recorded as swimming distance. Each animal was allowed a 120-second swim to find the platform, and stayed on the platform for a further 30 seconds. If the animal found the platform after the 120-second cutoff, escape latency was recorded as 120 seconds, and rats would be led to the platform by the experimenter. Spatial probe test was performed on day 5. The platform was removed and each animal was allowed to freely swim. The start position for each rat was opposite to the platform quadrant. The number of rats stepping across the original platform within 120 seconds was determined as cross platform frequency. The spatial probe test was carried out twice a day, for 4 days. The average score over the 4 days was used to determine the final behavioral performance of the rat. Escape latency and swimming distance in the place navigation test and the cross platform frequency in the spatial probe test were used to evaluate the animals’ learning and memory ability.

**Collection of specimens**

Rats were anesthetized with 10% chloral hydrate (300 mg/kg) via intraperitoneal injection, followed by intracardial perfusion with 0.1 mol/L PBS (pH 7.4) mixed with 4% paraformaldehyde at 30°C. The animals were killed by decapitation at the preset time points. The bilateral common carotid artery ligation was checked again. Frontal lobes were removed immediately on dry ice, wrapped with aluminum foil and then preserved in liquid nitrogen at −70°C. Serial coronal sections were cut from the frontal lobes and every section was 4 μm thick. One of every three sections was selected and mounted onto slides for staining.

**Immunohistochemical staining**

Briefly, the paraffin-embedded sections were dewaxed with xylene and dehydrated with a graded alcohol series. Subsequently, sections were incubated in 3% (w/v) H2O2 for 15 minutes, and washed with PBS three times for 5 minutes each. Then, antigen retrieval was carried out with 10 mmol/L sodium citrate buffer. The sections were treated with peroxidase for 10–15 minutes in blocking solution to block endogenous peroxidase, and then in 5% goat serum for 10 minutes to block non-specific antibody binding. Overnight incubation with rabbit anti-hypoxia-inducible factor-1α primary polyclonal antibody (1:100; Boster Bioengineering Limited Company, Wuhan, Hubei Province, China) and rabbit anti-rat heme oxygenase-1 primary monoclonal anti-body (1:100; Boster) was performed in humidified boxes at 4°C. PBS was used as a negative control. After that, tissue specimens were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2 000; Boster) at 37°C for 30 minutes, and with streptomycin avidin-peroxidase solution at 37°C for 30 minutes. Then, staining was developed with DAB solution for 5–10 minutes. Tissues were rinsed in PBS three times for 5 minutes each between each step, and then dyed in hematoxylin. Sections were subsequently mounted, dehydrated, coverslipped, and examined under an optical microscope (Olympus). Immunohistochemistry was analyzed with a HPLAS-1000 high-definition color pathology graphic analysis system (Olympus). Five different fields of view were selected randomly for each section. The number of positively-stained cells was the mean of five different fields of view.

**Semi-quantitative RT-PCR analysis**

The mRNA levels of hypoxia-inducible factor-1α and heme oxygenase-1 in the frontal cortex were measured using semi-quantitative RT-PCR. Primers (Sangon Bio-tech Co., Ltd., Shanghai, China) were designed according to the nucleotide sequences using Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Total RNA was extracted from tissues (50–100 mg) taken from the frontal lobe with Trizol. RNA concentration and...
purity were evaluated by spectrometry on the basis of optical density measurements at 260 and 280 nm. Using the extracted RNA as template, cDNA synthesis was performed in a 20-μL reaction mixture using reverse transcriptase. The reverse-transcribed products were preserved at −20°C until use. 1 μL cDNA from this mixture was used for RT-PCR amplification. The amplification conditions for hypoxia-inducible factor-1α were: pre-denaturing at 94°C for 2 minutes; 30 cycles of denaturing at 94°C for 20 seconds, annealing at 55°C for 30 seconds, extending at 72°C for 1 minute; final extension at 72°C for 5 minutes. Amplification conditions for heme oxygenase-1 and β-actin were similar to those for hypoxia-inducible factor-1α, except that the annealing temperature for heme oxygenase-1 was 53°C and the annealing temperature for β-actin (Sangon Biotech Co., Ltd) was 60°C (Table 1). The amplification products were quantified following 2% agarose gel electrophoresis. After scanning with a gel image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China), Bandscan (Tanon Science & Technology Co., Ltd.) was used to analyze band gray scale and to calculate the ratio of target gene band intensity to that of the corresponding β-actin band to determine the level of mRNA expression.

**Table 1** Primers and expected sizes of PCR products with each primer pair

| Gene          | Forward (5′→3′)       | Reverse (5′→3′)       | Size (bp) |
|---------------|-----------------------|-----------------------|-----------|
| HIF-1α        | GCT CCG CCA ACT       | GCT CCT GCC TCT       | 743       |
|               | CTCCCTGTC             | AGT CTC CAC C         |           |
| HO-1          | TTT CAC GTT CCC       | GCC TCT TCT GTC       | 111       |
|               | GAG CAT               | ACC CTG T             |           |
| β-actin       | CAC CGG CGT ACA       | CCC ATA CCC ACC       | 207       |
|               | ACC TTC               | ATC ACA C             |           |

HIF-1α: Hypoxia-inducible factor-1α; HO-1: heme oxygenase-1.

**Western blot analysis**

Frontal cortex tissues preserved in liquid nitrogen were rapidly ground, followed by PBS washing and centrifugation (centrifugal radius 13.5 cm) twice, at 1 000 r/min for 5 minutes. Then, the cell pellet was topped with 150 μL cell lysis buffer (including 50 mmol/L Tris-Hcl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 0.5 sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 0.5 mmol/L dithiothreitol, 1–2 μg/mL aprotinin). Cell lysates were homogenized for 30 minutes at 4°C and centrifuged (centrifugal radius 9.35 cm) at 12 000 r/min for 2 minutes at 4°C. The supernatants obtained were saved and used as the total protein extract. Protein concentrations were quantified with the Biorad protein assay and stored at −20°C. Protein samples were separated on SDS-PAGE mini gels at 120 V until bromophenol blue reached the bottom of the separation gel. Equivalent amounts of total protein for each sample were loaded (20 μL). They were subsequently transferred electrophoretically to a nitrocellulose membrane by applying a 110 V current at 4°C for 1.5 hours. The membrane was immersed in PBS for 12 minutes and then stained with Ponceau stain for 12 minutes. After blocking with 5% skimmed milk powder for 3 hours at room temperature, the membrane was hybridized with rabbit anti-hypoxia-inducible factor-1α primary polyclonal antibody and rabbit anti-heme oxygenase-1 primary polyclonal antibody (Boster) diluted in 0.2% Tris-buffered saline solution overnight at 4°C. Afterward, they were washed with Tris-buffered saline solution, three times for 10 minutes each, at room temperature and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary IgG (Santa Cruz Biotechnology, Texas, USA; 1:2 000; 0.1 mL/cm²) at 4°C for 1 hour. The nitrocellulose membranes were washed with Tween 20-Tris buffer salt solution, twice for 5 minutes each, and then with Tris-buffered saline solution, once for 10 minutes. Immunoreactive bands were visualized with 3,3′-diaminobenzidine. Protein bands were quantified by image analysis with Bandscan 5.0 (Tanon Science & Technology Co., Ltd.).

**Flow cytometry analysis**

Nine weeks after chronic cerebral ischemia, the number of apoptotic cells in the frontal lobe was determined by flow cytometry (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Quantitative analysis of apoptosis was based on the accumulation of various sized DNA oligonucleotide fragments, which was displayed as the diploid apoptosis peak before the G1 peak in the flow cytometry DNA histogram. G0/G1, S, G2/M phase cell percentage, and apoptotic cell percentage were shown in the flow cytometry report.

**Statistical analysis**

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA), and were expressed as mean ± SD. Difference was considered significant at P < 0.05, which was assessed using one-way analysis of variance.

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