

Changes in Levels of Hypoxia-Induced Mediators in Rat Hippocampus During Chronic Cerebral Hypoperfusion

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Abstract The hypoxia-inducible factor (HIF)-mediated signaling pathway is an adaptive and protective mechanism that is triggered by hypoxia, ischemia, and other pathophysiological conditions. The expression of HIF-1 α and downstream genes, some of which are pro-apoptotic whereas others are pro-survival, is up-regulated in ischemic stroke. Interestingly, however, the effects of HIF-1 α activation are different in the early and late stages of acute cerebral ischemia, and these differences may depend on the duration and severity of hypoxia. Therefore, in the present study, we investigated the effect of HIF-1 α activation in chronic cerebral hypoperfusion, which plays an important role in the development of dementia. Permanent bilateral common carotid artery occlusion (2VO) was used to induce chronic global cerebral hypoperfusion in rats. The expression of HIF-1 α protein and the transcription of downstream genes were measured at different time points, including 0 h, 12 h, 24 h, 3 days, 7 days, 14 days, 28 days, 42 days, and 56 days after 2VO. HIF-1 α increased as early as 12 h after the occlusion and remained high for at least 56 days. Interestingly, mRNA levels of both pro-apoptotic (Bcl-2/adenovirus E1B 19 kDa-interacting protein 3, NADPH oxidase activator 1, and NIP3-like protein X) and pro-survival (vascular endothelial growth factor, glucose transporter-1) genes were up-regulated at the early stage after 2VO, followed by a gradual decline to baseline/control levels. Thus, HIF-1 α increased consistently during chronic cerebral hypoperfusion, whereas both pro-apoptotic and pro-survival downstream genes were up-regulated only early after 2VO. This mismatch in gene expression

may contribute to the lack of a protective effect of highly expressed HIF-1 α during the chronic stage of cerebral hypoperfusion.

Keywords Chronic global cerebral hypoperfusion · Hypoxia-inducible factor · Pro-apoptotic and pro-survival genes

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a pivotal regulator of gene expression in response to hypoxia. HIF-1 target genes include pro-survival genes involved in angiogenesis, energy metabolism, erythropoiesis, vasomotor control, and cell proliferation, such as vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), and erythropoietin (EPO) [1, 2]. Paradoxically, HIF-1 α is also involved in hypoxia-dependent inflammation, apoptosis, and cellular stress. Some of the pro-death genes that are involved include Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP-3), NADPH oxidase activator 1 (Noxa), NIP3-like protein X (NIX), and others [3–5]. Consequently, despite the undisputed role of HIF-1 in regulating oxygen homeostasis, both neuroprotective and detrimental effects of HIF-1 have been observed in various acute ischemia models [6]. Some research has indicated a role for HIF-1 α in mediating both pro-death and pro-survival responses that may depend on the duration of the ischemia [7] and the types of pathological stimuli [8]. Nevertheless, whether elevated HIF levels are detrimental or protective in the setting of cerebral ischemia and what role the time course of HIF activation and downstream gene effects play are unclear.

Chronic cerebral hypoperfusion (CCH), a preclinical condition of mild cognitive impairment and the prevalent

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pathophysiological state in patients with dementia [9], is a less severe form of ischemia that develops more slowly than an acute ischemic stroke [10, 11]. A number of studies have identified cerebral hypoperfusion in brain regions associated with cognitive functions both in Alzheimer's disease and in vascular dementia patients, and have supported the notion of a reduced blood supply to brain as a decisive factor in the pathogenesis of cognitive impairment [12, 13]. Therefore, HIF-induced long-term effects may be important factors in the development of CCH, which may provide a potential target for dementia therapy.

In this study, we examined HIF-induced effects during CCH. Permanent bilateral common carotid artery occlusion (2VO) was used to induce the rat model of chronic global cerebral hypoperfusion. The expression of HIF-1 α and the effects on transcription of downstream pro-survival and pro-apoptotic genes were measured for 8 weeks after the vessels were ligated.

Materials and Methods

Animals

120 Male Wistar rats (weight 220–250 g, 7 weeks old, obtained from Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were group-housed in polypropylene cages at 22 ± 2 °C with 55 ± 5 % humidity and maintained on a 12-h dark/light artificial cycle (lights on at 7:00 a.m.) with ad libitum access to water and food. All animal procedures were performed according to protocols approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Ethics Committee of the Medical School of Wuhan University approved their use.

Model Establishment and Experimental Design

Chronic cerebral hypoperfusion was induced with 2VO [14]. Food and water were withheld for 1 day prior to surgery. Rats were anesthetized with 10 % chloral hydrate (300 mg/kg, i.p.). The bilateral common carotid arteries of rats were exposed via a midline ventral incision, carefully separated from the vagal nerves, and permanently ligated with silk suture. Rats that underwent a sham operation were treated in a similar manner, except that the common carotid arteries were not ligated. During the operation, body temperature was maintained at 37.5 ± 0.5 °C. After surgery, the wounds were sutured, and rats were allowed to recover from anesthesia before being returned to their cages.

The levels of various hypoxia-induced mediators were detected with quantitative real-time PCR (qPCR) and Western blotting at different time points, including 0 h (before

2VO, without occlusion), 12 h, 24 h, 3 days, 7 days, 14 days, 28 days, 42 days, and 56 days after 2VO. Samples for detection were obtained from the hippocampus of rats after they had been anesthetized with chloral hydrate. The tissue was frozen immediately in liquid nitrogen and stored at -80 °C until Western blotting and qPCR.

qPCR

Total RNA was extracted from individual hippocampi using the Trizol method (Invitrogen). RNA (4 μ g) was reverse transcribed into cDNA using a First Strand cDNA Synthesis kit (TOYOBO, Japan). cDNA (0.2 μ g) was amplified with a qPCR kit using the gene-specific primers listed in Table 1. The reaction was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). The PCR cycles were as follows: initial denaturation (50 °C, 2 min; 95 °C, 2 min), 40 cycles (95 °C, 15 s; annealing at 58 °C, 15 s; extension at 72 °C, 45 s), and a final extension (72 °C, 10 min). Specific amplification was confirmed with melting curve analysis. All reactions were performed in triplicate using β -actin as an endogenous control. The changes in mRNAs were expressed as fold-change using the formula ($2^{-\Delta\Delta C_t}$).

Western Blotting

Nuclear proteins were extracted from the hippocampus of rats for immunoblotting analysis using a kit from the Keygen Institute of Biotechnology (Keygen, Nanjing, China). Protein samples (40 μ g) were fractionated with 10 % SDS-PAGE, transferred to 0.45- μ m PVDF membranes at 200 mA for 1 h, and stained with Ponceau red to confirm equal loading. The blots were blocked with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween 20 (TBST) at room temperature for 1 h. Membranes were then incubated with primary antibodies (anti-HIF-1 α : 1:1,000 in TBST, BD Transduction Laboratories, San Diego, CA, USA; anti- β -actin: 1:2,000 in TBST, Santa Cruz, Dallas, TX, USA) at 4 °C overnight, washed three times with TBST, and incubated with peroxidase-conjugated secondary antibodies (anti-mouse: 1:3,000 in TBST, KPL, MD, USA) for 30 min. Membranes were washed three times with TBST, and labeled proteins were visualized with chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA). Semiquantification of immunoreactive bands on X-ray film was performed with optical density analysis (HPIAS 2000, Tongji Qianping Company, Wuhan, China). Densities were normalized to β -actin.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5.0 software. Data are the mean \pm SEM. The Western blot

Table 1 Primers used for qPCR analysis

Gene	Orientation	Sequence (5'–3')	Amplicon size (bp)
VEGF	Forward	TGTGAGCCTTGTTCAGAGCG	252
	Reverse	GACGGTGACGATGGTGGTGT	
EPO	Forward	CACCCTGCTGCTTTACTATCCTT	139
	Reverse	CATTGTGACATTTTCTGCCTCCT	
GLUT-1	Forward	CCGCTTCCTGCTCATCAATC	122
	Reverse	TCATCTGCCGACCCTCTTCT	
BNIP-3	Forward	ATGGGATTGGTCAAGTCGGC	205
	Reverse	CTTCCAATGTAGATCCCCAAT	
Noxa	Forward	ACGTGGAGTGCACCGGACAT	168
	Reverse	TTTCTGCCGTAAATCACTT	
NIX	Forward	TTCAGACACCCTAAGCGTGC	176
	Reverse	GCAGAAGGTGTGCTCAGTCGT	
β -actin	Forward	CGTTGACATCCGTAAAGACCTC	110
	Reverse	TAGGAGCCAGGGCAGTAATCT	

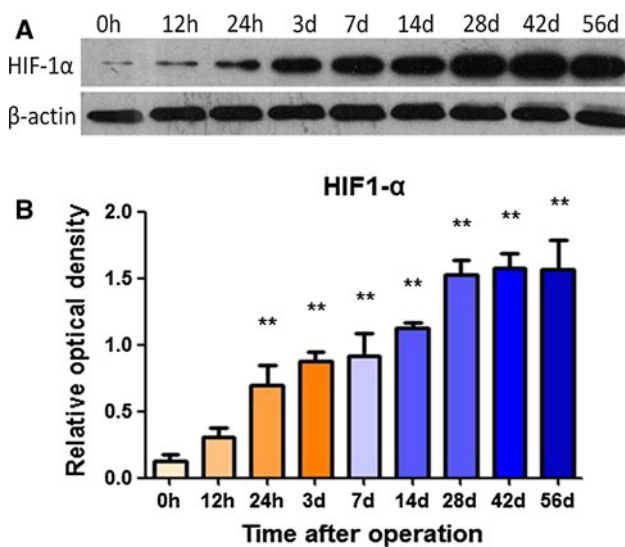


Fig. 1 Western blot analysis of HIF-1 α expression in rat hippocampus at different times (0 h–56 days) after 2VO operation. **a** Representative Western blotting result from hippocampal nucleoprotein extracts probed with the indicated antibodies. **b** Quantitation of HIF-1 α protein expression. Each *bar* represents the mean relative optical density \pm SEM ($n = 6$). ** $p < 0.01$ as compared with 0 h

results were analyzed with one-way ANOVA with Dunnett's test. PCR results were analyzed with two-way ANOVA followed by Bonferroni post-tests. $p < 0.05$ was considered statistically significant.

Results

Accumulation of HIF-1 α During CCH

To determine whether HIF-1 α accumulated in response to chronic global hypoperfusion, the expression of HIF-1 α in the

hippocampus at different time points after 2VO was examined with Western blotting. Chronic cerebral hypoperfusion induced by 2VO triggered a steady increase in HIF-1 α levels in the hippocampus (Fig. 1). Protein levels began to increase as early as 12 h after the operation ($p > 0.05$; Fig. 1b), were increased by more than 10-fold by 28 days, and remained high for at least 56 days ($p < 0.01$; Fig. 1b).

Transcription of HIF-1–Mediated Pro-Survival Genes During CCH

Vascular endothelial growth factor, GLUT-1, and EPO are genes with established roles in neuronal survival and/or vascular biology, and their expression is regulated by HIF-1. qPCR analysis showed that mRNA levels of VEGF and GLUT-1, but not EPO, were significantly increased in the hippocampus in the early stage after 2VO (Fig. 2). VEGF mRNA peaked at ~ 24 h–3 days ($p < 0.01$; Fig. 2a) and then declined slowly to normal levels. Similarly, the expression of GLUT-1 increased significantly as early as 12–24 h after 2VO ($p < 0.01$) and remained higher in the first 7 days as compared with sham-operated rats ($p < 0.01$, $p < 0.05$; Fig. 2b). In contrast, the expression of EPO was a little higher than that in sham rats, but the differences were not statistically significant over the entire time period that we examined ($p > 0.05$; Fig. 2c).

The Transcription of HIF-1–Mediated Pro-Apoptotic Genes During CCH

Similar to pro-survival genes, the expression of HIF-1–regulated pro-death genes (BNIP-3, NIX, Noxa) tended to increase in the early stage after 2VO (Fig. 3). The induction of NIX and Noxa at the mRNA level peaked at 3 days after the vascular ligation ($p < 0.05$, $p < 0.01$; Fig. 3b, c) and

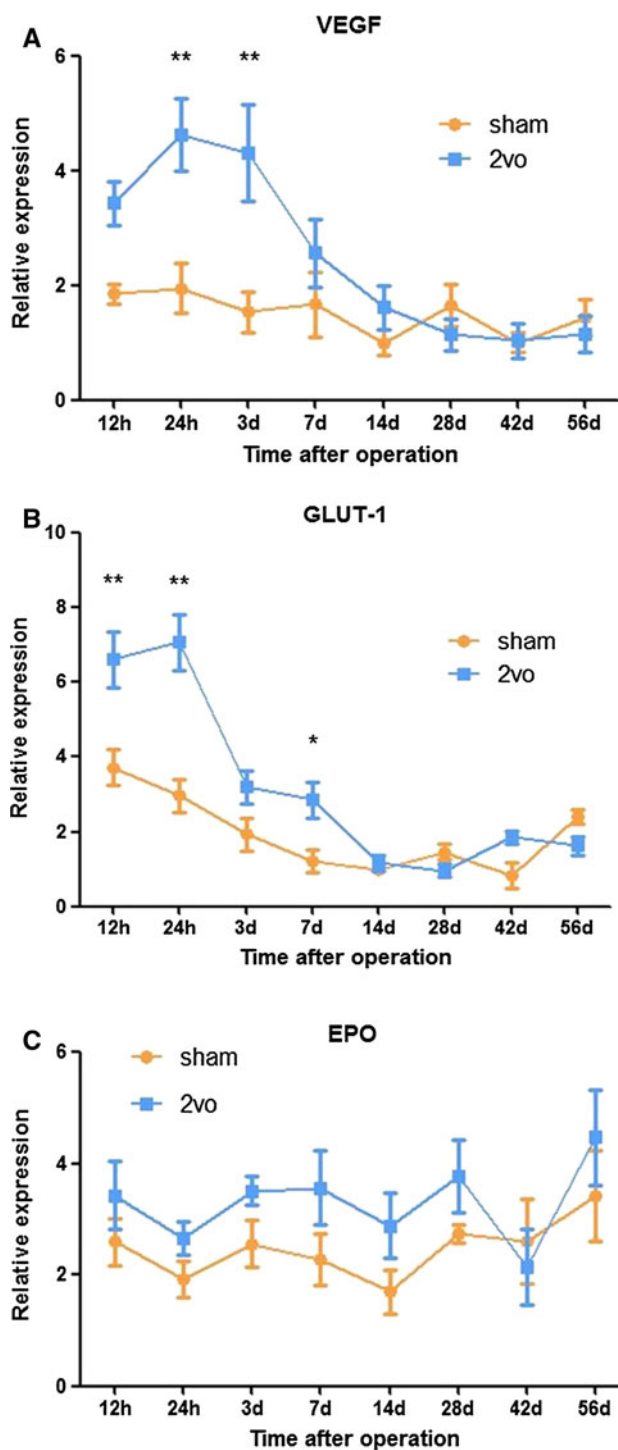


Fig. 2 HIF-1-dependent expression of pro-survival genes in rat hippocampus during CCH. qPCR analysis of the indicated genes (a VEGF; b GLUT-1; c EPO) at different times after 2VO or sham operation. Values represent the mean relative expression \pm SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$ as compared with sham-operated rats

then decreased to non-hyperperfused levels. The expression of BNIP showed similar increasing trend in the first three days, but without statistical significance ($p > 0.05$, Fig. 3a).

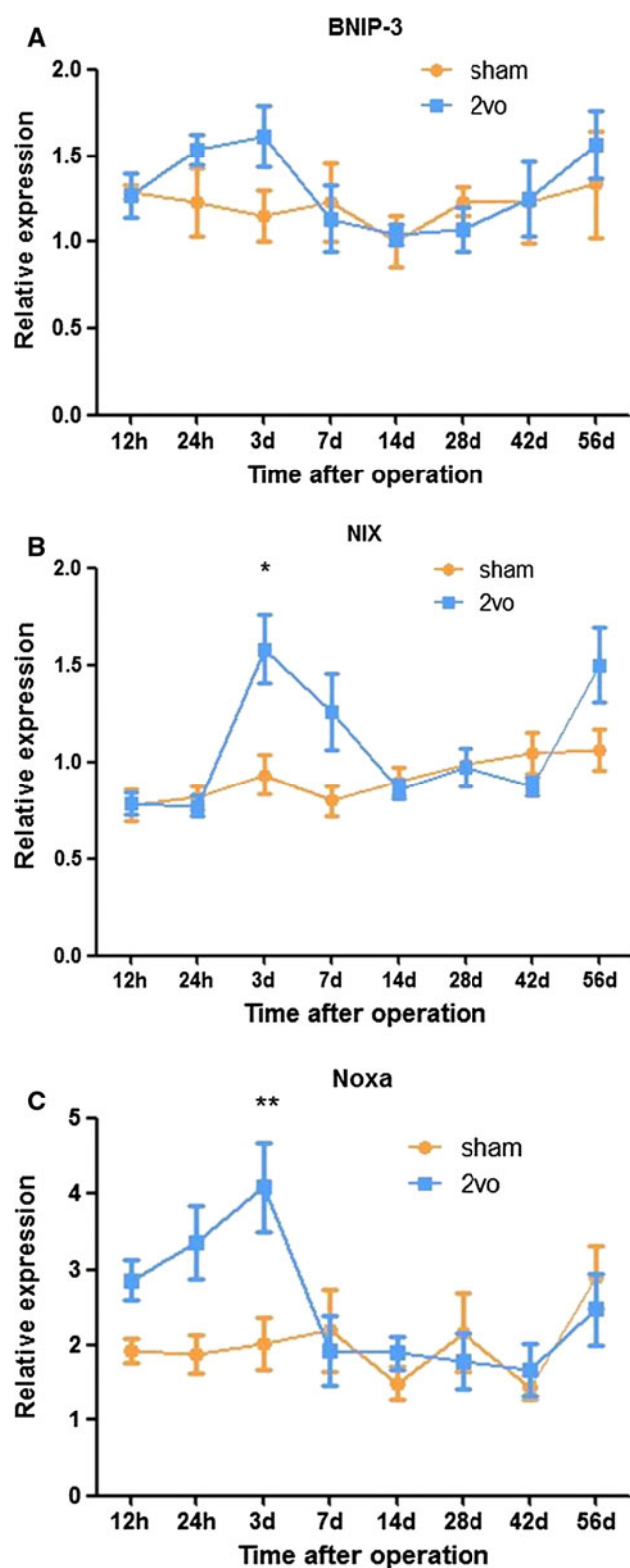


Fig. 3 HIF-1-dependent expression of pro-apoptotic genes in rat hippocampus during CCH. qPCR analysis of the indicated genes (a BNIP-3; b NIX; c Noxa) at different times after 2VO or sham operation. Values represent the mean relative expression \pm SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$ as compared with sham-operated rats

Discussion

Increasing number of studies indicate that cerebral hypoperfusion is one of the initial conditions that are critical to the development of cognitive dysfunction [15]. Cerebral hypoperfusion can cause disorders in neuronal electrical activity, inhibition of protein synthesis, apoptosis, and oxidative stress, which may be pathophysiological mechanisms that contribute to cognitive impairment [16]. Despite the detrimental effects of hypoperfusion, the decline in cellular oxygen levels does, however, induce compensatory or endogenous adaptive and regenerative mechanisms to defend against hypoperfusion injury and to allow recovery of brain function [16, 17]. Regulation of the endogenous adaptive processes involves the concerted activation of various transcription factors, including HIF. As our data show, chronic global cerebral hypoperfusion triggered long-lasting accumulation of HIF-1 α protein in rat hippocampus, which is a brain region that is susceptible to ischemia and is involved in cognition.

HIF-1 is a dimeric transcriptional complex consisting of HIF-1 α and HIF-1 β subunits. HIF-1 activity is controlled by the availability and activity of the HIF-1 α subunit. Under normoxic conditions, HIF-1 α is hydroxylated by oxygen-dependent proline hydroxylases (PHDs) and is rapidly degraded. Under hypoxic-ischemic conditions in which oxygen is limited, PHDs are less active, and, thus, the HIF-1 complex becomes stabilized and accumulates in the cytosol. The HIF-1 complex then translocates to the nucleus, resulting in transcription of HIF-1 target genes such as VEGF, EPO, GLUT-1, etc., which promote neuronal adaptation for survival under hypoxic conditions [2]. These downstream genes are responsible for protection from cerebral ischemia/hypoxic injury [1]. The expression of VEGF and EPO activates VEGF receptors and EPO receptors, respectively, which promote vasodilation and angiogenesis, inhibit excitotoxicity, and protect neural cells from apoptosis [18, 19]. GLUT-1 is highly expressed in brain endothelial cells and is critical for glucose entry into the CNS. GLUT-1 enhances the concentration of glucose and thereby also promotes neuroprotection [20].

HIF-1 α protein accumulates and there is a consequent up-regulation of HIF-1 target genes (VEGF, EPO) after cerebral ischemia [21, 22]. Chavez and LaManna showed that HIF-1 α accumulation starts as early as 1 h after restoration of blood flow and persists up to 7 days after transient global ischemia [23]. Neuron-specific knock-down of HIF-1 aggravates brain damage after a 30-min middle cerebral artery occlusion (MCAO) and reduces the survival rate of MCAO mice [24]. Inhibiting HIF-1 PHDs to trigger the accumulation of HIF-1 α protects the brain from cerebral stroke damage [25, 26]. Thus, studying HIF-1 appears to be important for the development of

novel therapeutic interventions in ischemia-related neurological disease.

As CCH is less severe and more slowly developing global ischemia, HIF-1A may play a more important role in the pathological process. Unfortunately, how it works is still poorly understood. In the present study, we revealed that similar to acute ischemia, after 2VO, HIF-1 α levels increased initially and remained elevated during hypoperfusion for at least 8 weeks. The expression of the HIF-regulated genes VEGF and GLUT-1 was higher in the first 7 days as compared with expression in sham-operated rats. The expression of EPO was slightly higher than in the sham group.

Previous researchers have noted an increased capillary diameter, neovascularization, and enhanced VEGF immunohistochemistry in the cortex and hippocampus in models of CCH, as well as enlargement of the arteries at the base of the brain, and these events coincide with the upregulation of HIF-mediated genes [27, 28]. In addition, cerebral blood flow values gradually begin to recover at 1 week and normalize over a period of months in the 2VO model, even though the occlusion is permanent [17, 29]. Under these conditions, compensatory or adaptive mechanisms are turned on. Thus, we hypothesize that the HIF transcription complex may be involved in endogenous protective mechanisms during CCH by activating the genetic program of hypoxic adaptation.

HIF-1 has also been shown to mediate pro-death responses, although its probable role in triggering adaptive responses has been detected. HIF governs the expression of several pro-apoptotic genes including BNIP-3, NIX, and Noxa [30], which cause mitochondrial dysfunction leading to cell injury and death [3–5]. The expression of HIF-1-regulated pro-death BH3-only family members such as BNIP-3 is increased after cerebral ischemia [31, 32]. Brain-specific knockout of HIF-1 α may be neuroprotective after acute global ischemic injury for 75 min [33]. Likewise, our data revealed significant increases in NIX and Noxa transcription on the third day after 2VO. Consequently, HIF-1 α appears to mediate both pro-death and pro-survival responses during CCH.

Which of the HIF-1 α -related functions is most dominant may depend on the severity or duration of the ischemic or hypoxic insult [7, 8] as well as the cell type in which it is induced [34]. In a rat model of focal ischemia, selective inhibition of HIF-1 α during early, but not late, time points was neuroprotective [35]. In addition, the different effects of transient focal ischemia for 30 min [24] and acute global ischemia for 75 min as described above [32] support the dual characteristics of HIF.

Considering the increase in expression of both pro-survival and pro-apoptosis genes during early stages of CCH, whether HIF-mediated pro-survival responses are dominant

over deleterious effects or vice versa remains unknown. Our results suggest that HIF-regulated pro-survival genes changed more than pro-death genes. The tendency of an increase in pro-survival genes was sustained for at least 1 week, whereas the increase in pro-apoptosis genes was relatively temporary. We will next perform research in which the accumulation of HIF-1 is blocked or promoted to explore its exact function.

It is worth mentioning that, mismatching with the expression of HIF-1 α , the level of downstream genes display no significant difference between 2VO and sham operation rats at the late stage of hypoperfusion, which might attribute to the lack of sufficient protective effect of highly expressed HIF-1 α level at the chronic stage.

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Conflict of interest The authors declare no competing financial interests.

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