Hypoxia-Inducible factor-1α promotes neuroregeneration and angiogenesis after cerebral ischemia

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To promote neuroregeneration and angiogenesis is a therapeutic target for the treatment of stroke. Hypoxia-inducible factor-1α (HIF-1α) has pleiotropic effects on neurogenesis, angiogenesis and neuroprotection in central nervous system. In this study we investigated whether HIF-1α treatment promotes the proliferation of ischemia induced neural stem cells (NSCs) and neuroregeneration in penumbra. The angiogenesis in penumbra was also investigated. Transient middle cerebral artery occlusion (tMCAO) rat model was used in this study. Rats were divided into 3 groups, Sham group, vehicle group and HIF-1α group. In this study, we found that rats with HIF-1α treatment had better behavioral recovery at day 7, 14, 21 and 28 (p<0.05). HIF-1α treatment increased the number of ischemia induced endogenous NSCs in penumbra obviously (p<0.01). HIF-1α also improved newborn neurons and glial cells in penumbra on the 28 d (p<0.01). HIF-1α treatment promoted expression of erythropoietin (EPO) in peri-ischemic area. Vascular endothelial growth factor (VEGF) expression in HIF-1α group was significantly higher than that in vehicle group (p<0.01). Number of CD 31 positive vascular in penumbra in HIF-1α treatment group was much more than that in vehicle group (p<0.01). In conclusion, our results indicate that modulate HIF-1α after ischemia may be a therapeutic target for the treatment of ischemic stroke through promoting neuroregeneration and angiogenesis.

Keywords: hypoxia-inducible factor-1α; cerebral ischemia; neuroregeneration; angiogenesis; rat

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Introduction

Neurovascular unit is constituted by the capillary endothelial cells, neurons, the site of anatomical blood-brain barrier, and nonneuronal cells such as astrocytes, pericytes, and microglia. It is essential for the health and function of the central nervous system (CNS). Improving the function of neurovascular unit protected against ischemic injury [1]. Neurovascular unit can be a therapeutic target for stroke.

After ischemic stroke, there is some tissue which is still salvageable surrounding the core of infarction. This tissue is ischemic penumbra. Ischemic penumbra is a main target of treatment for ischemic stroke [2,3]. One of the key methods to save the cells in ischemic penumbra is to improve angiogenesis or neurogenesis. Recent studies showed that neurogenesis and angiogenesis are linked to each other [4].

HIF-1α, a subunit of HIF-1, is degraded rapidly by 26s proteasomes in normoxia. During hypoxia, it is stabilized and transactivated to the nucleus. HIF-1α target genes include EPO, VEGF, several glycolytic enzymes and stromal-derived growth factor1. These target genes have pleiotropic effects on neurogenesis, angiogenesis, and neuroprotection in the
brain [5-8]. HIF-1α plays pivotal roles in the early development of brain and many other mammalian organs [9]. Under normal conditions, overexpression of HIF-1α increases the number of TH-positive cells and the dopamine content in culture medium [10]. Knock out HIF-1α in mice show severe cardiac and vascular malformations, leading to embryonic lethality at embryonic day 10.5. HIF-1α conditional knock-out midbrain-derived neural precursor cells show midbrain-specific impairment of survival and proliferation [11].

It has been shown that HIF-1α gene therapy enhances capillarization in rat myocardial infarction model, and then increases regional blood flow and reduces the infract size [12]. Inactivation of endogenous HIF-1α by dimethyloxalylglycine promotes angiogenesis in ischemic skeletal muscles of mice [13]. Inoculating HIF-1α DNA into the brain surface or the temporal muscle promotes significant angiogenesis development in a rat encephalo-myo-synangiosis (EMS) model [14]. In skeletal muscle, stabilized HIF-1α increases capillary sprouting and proliferation obviously, whereas VEGF only induces proliferation of endothelial cells without forming of proper capillary structures. Furthermore, unlike that of VEGF, the overexpression of HIF-1α does not increase vascular leakiness in the transduced muscle [15]. These results suggest that increasing HIF-1α activity may be more efficacious than only increasing a single factor.

Previous study found that there is a significant increasing of HIF-1α expression in penumbra after ischemic stroke. The aim of this study is to investigate whether HIF-1α gene can promote neuroregeneration and angiogenesis in a rat model of focal cerebral ischemia.

Materials and Methods

Rats

Male Sprague-Dawley rats, weighing 230-250 g, were used in this study. Animals were housed individually and maintained with room temperature of 25°C and had free access to food and water on the condition of a 12-hour light-dark cycle before surgery.

Transient MCAO model

Rats were initially anesthetized with 50 mg/kg of ketamine and 10 mg/kg of xylazine administered intraperitoneally. We induced transient MCAO according to the method of intraluminal vascular occlusion [16, 17]. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were isolated. A 3 cm length of 4-0 nylon suture with a slightly enlarged and rounded tip was advanced from the ECA into the lumen of the ICA until it blocked the origin of the middle cerebral artery (MCA). The distance from the tip of the suture to the bifurcation of the common carotid artery is 18.5 to 19.5 mm. Reperfusion was performed by withdrawing the suture until the tip cleared the lumen of the ECA after 90 min of MCAO.

Treatment procedures

Recombinant adenovirus with HIF-1α gene (Ad-HIF-1α) was constructed using AdEasy system in Chinese academy of science. The resultant titer of Ad-HIF-1α was between 10⁹ and 10¹⁰ pfu/ml. After MCAO, rats were randomly divided into three groups immediately (n=30): Sham group (n=10), ischemia+vehicle (vehicle group, n=10), ischemia+Ad-HIF-1α (HIF-1α group, n=10). Using aseptic technique, a 2 to 5 mm incision was made in the scalp 1.5 mm lateral to the bregma. A burr hole was made in the bone 3 mm posterior to bregma with a dental drill, and Ad or Ad-HIF-1α of about 10 μL was slowly injected into the ischemic area 2.5 mm deep from the surface of the brain in 30 min. The needle was retained in the brain for an additional 5 min before retraction.

Behavioral evaluation

Behavioral testing was performed weekly using the modified neurological severity score (NSS) [18]. The NSS comprises motor (muscle status, abnormal movement), sensory (visual, tactile, proprioceptive), reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18), higher score, more severe injury.

BrdU Labeling

To evaluate the number of ischemia induced endogenous NSCs in penumbra, BrdU (50 mg/kg) was administered intraperitoneally every 4 hours for 3 times. Rats were sacrificed 4 hours after the last injection.

At days 7 and 28 after tMCAO, the rats were terminally anesthetized by pentobarbital (60 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde. All brains were dissected and postfixed in the same fixative overnight at 4°C. After fixation, brains were processed for paraffin sections (5μm). Paraffin sections were
deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 12-15 min in PT module. Sections were incubated in 0.3% H2O2 in 50% methanol for 30 min at room temperature to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in 3% BSA for 30 min and then a biotin blocking system (Dako) was used to block endogenous biotin. Sections were then incubated with anti-EPO (1:200; Abcam), anti-CD31 (1:100; Abcam), and at 4°C after blocking nonspecific binding in 3% BSA. BSA replaced primary antibodies in negative controls. After washing, sections were incubated with HRP polymer kit (Biocare Medical; GHP516) for 30 min at room temperature, followed by 3, 3′-diaminobenzidine tetrahydrochloride as the chromogen. For immunofluorescence, steps for quenching of endogenous peroxidase and blocking of endogenous biotin were omitted. Sections were incubated overnight with anti-BrdU (1:400; Boster), anti-NF-200(1:100; Boster), anti-GFAP (1:500, Abcam), anti-EGFR (1:100 Santa Cruz) at 4°C after blocking nonspecific binding in 3% BSA. Primary antibodies were detected with donkey anti goat Cy3 (1:400; Jackson Immuno Research), donkey anti rabbit FITC (1:400; Santa Cruz Biotechnology), donkey anti mouse FITC (1:400; Jackson Immuno Research). Sections were later counterstained with Vectashield mounting medium containing 4′, 6′-diamidino-2-phenylindole (DAPI) (Vector) to label nuclei (43). Ten sections were investigated per rat and positive cells were counted in ×400 or ×200 power electronmicroscopic fields for each section.

Statistical analysis

All data were presented as mean ± SD. The data were analyzed by Student’s t-test. A probability value of less than 0.05 was considered statistically significant.

Results

Rats treated with HIF-1α had better behavioral recovery

No significant difference was found in NSS scores between the vehicle group and HIF-1α group before tMCAO or on day 1 after tMCAO. In both groups, behavioral deficits evaluated by the NSS demonstrated a progressive recovery from day 7 to day 28 after MCAO. Rats in HIF-1α group showed a significant better improvement in NSS score compared with Ad group on day 7, 14, 21, 28 following ischemia (Fig. 1). In sham group, no behavioral deficits were observed. Body weights, food intake between the 3 groups during the 4 weeks were no significant difference.

HIF-1α treatment increased EPO expression after ischemia

EPO expresses mostly in cytoplasm. It can be found in nucleus. A few EPO positive cells were found in the cortex in sham group (Fig. 2 A). On day 7 after tMCAO, EPO expression slightly increased in peri-ischemic area in vehicle group (Fig. 2 B). It was significantly higher after treated with HIF-1α (Fig. 2 C). No significant difference was found between sham group and vehicle group. The difference was significant between vehicle group and HIF-1α group (Fig. 2 D)
Table1. Comparing the number of BrdU, BrdU/NF200 and BrdU/GFAP in peri-ischemia area (×200 field)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BrdU</th>
<th>BrdU/NF200</th>
<th>BrdU/GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>1.15± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ad</td>
<td>5</td>
<td>23.38±4.35</td>
<td>5.73±1.81</td>
<td>10.17±2.72</td>
</tr>
<tr>
<td>Ad-HIF-1α</td>
<td>5</td>
<td>61.57±8.26</td>
<td>12.42±2.73</td>
<td>48.25±5.44</td>
</tr>
</tbody>
</table>

Differentiation of proliferating cells in the peri-ischemia area. At 28 d after ischemia, proliferating cells differentiate into neuron-like cells (BrdU/NF200) or glial-like cells (BrdU/GFAP). *p<0.01, vs Ad group

Fig 2. Expression of EPO in each group on day 7 after tMACO. There are a few EPO positive cells in cortex in sham group. EPO expression slightly increased in peri-ischemic area in vehicle group. It is significant higher after treated with HIF-1α. (* p < 0.05)

HIF-1α treatment promoted proliferation and differentiation of ischemia induced endogenous NSCs

To determine if HIF-1α promotes proliferation of endogenous NSCs in peri-ischemic area, rats were injected with BrdU. The number of endogenous NSCs was detected by BrdU immunofluorescence staining on day 7 after ischemia. BrdU positive cells were rare in sham group (Fig. 3A). In vehicle group, ischemic induced endogenous NSCs were found in peri-ischemic area (Fig. 3 B). There were much more BrdU-positive cells in HIF-1 α group (Fig. 3 C). Quantitative analysis indicated that the number of endogenous NSCs in HIF-1α group was significantly increased than that in Ad group (Fig. 3 D).

On day 28 after ischemia, BrdU-positive cells were still found in peri-ischemia area. Double-label immunofluorescence demonstrated that newborn cells differentiated into neurons (BrdU/NF200) and glial cells (BrdU/GFAP). The number of BrdU-positive cells, BrdU/NF200 positive cells and BrdU/GFAP positive cells in HIF-1α group significantly increased compared with that in vehicle group (Table 1).

VEGF expression was increased in HIF-1α group

To evaluate if HIF-1α treatment increases the expression of VEGF, VEGF immunofluorescence was performed at day 7 of after ischemia. VEGF was found in endothelial cells in
Expression of VEGF was sharply decreased on day 7 after ischemia. It indicated that endothelial cells were damaged by ischemia. However, expression of VEGF was almost the same as in sham group when mice were treated with HIF-1α. (Fig. 4).

HIF-1α treatment improved the number of CD31 positive cells in peri-ischemia area

To determine if Ad-HIF-1α promoted proliferation of endothelial cells in the lesion sites, the number of endothelial cells was detected by CD31 immunostaining on day 28 after ischemia. VEGF was found in endothelial cells in the three groups. The number of CD31-positive cells decreased on day 28 after ischemia. When tMACO mice were treated with HIF-1α, the number of CD31-positive cells was similar to that in sham group (Fig. 5). Quantitative analysis showed that the number of CD31-positive cells in HIF-1α group significantly increased compared with that in vehicle group (Fig. 5 D).

Discussion

To test the hypothesis that HIF-1α contributes to both neurogenesis and angiogenesis following cerebral ischemia, in this study tMCAO rats were treated with recombinant adenovirus with HIF-1α gene. The results showed that rats treated with HIF-1α had better behavioral recovery. HIF-1α treatment promoted both neurogenesis and angiogenesis. And increased expression of EPO and VEGF by HIF-1α may
be the potential mechanisms of neurogenesis and angiogenesis.

In this study, BrdU was used as an indicator of neurogenesis. We confirmed the differentiation of BrdU-labeled cells by BrdU/NF200 double staining and BrdU/GFAP double staining. The results showed that HIF-1α improved both proliferation of endogenic NSCs and differentiation of these cells. Neurogenesis is a process through which precursor cells differentiate toward a mature neuronal phenotype, persists in discrete regions of the adult brain, including the rostral subventricular zone of the hippocampal dentate gyrus and the subgranular zone (SGZ). In adult brain neurogenesis induced by ischemia might be an important therapeutic target for ischemic stroke.

It has been shown that activation of HIF-1α could increase the expression of several key target genes such as erythropoietin, VEGF, tyrosine hydroxylase and OCT4, which act as regulators of cellular proliferation and differentiation [19]. Previous study showed that both EPO and its derivate CEPO treatment increased the number of (NeuN) BrdU/Neuronal Nuclei double-labeled cells in the dentate gyrus. It also showed EPO treatment improved spatial and non-spatial recognition memory in adult healthy mice [20]. In adult hippocampal neural progenitor cell cultures, EPO treatments increased the lengths of neurites when continuously applied for five days during differentiation [21]. In neural progenitor cells EPO promoted VEGF secretion through ERK1/2 and PI3K/Akt signaling pathways. Neural progenitor cells treated with rhEPO upregulated expression of VEGFR2 in endothelial cells, which along with VEGF secreted by neural progenitor cells promote angiogenesis [22]. This study showed that EPO expression after ischemia was significantly increased by HIF-1α treatment. EPO may be one of the possible mechanisms of promoted neurogenesis by HIF-1α treatment. The exact mechanisms about how HIF-1α promotes neurogenesis needs to be further researched.

In our study angiogenesis after ischemia was also evaluated. The results demonstrated that HIF-1α treatment promoted angiogenesis in peri-ischemic area after ischemia. HIF-1α, a universal molecular master switch, controls glucose metabolism and transport, cellular survival and metabolic adaptation [23]. Approximately 30 target genes are known to be regulated by HIF-1α, including VEGF, glycolytic enzymes glucose transporter 1, EPO and insulin-like growth factor 2 etc [24]. VEGF is the most well-known angiogenetic factor in each step of angiogenesis [25]. It regulates recruiting endothelial cells into hypoxic and

Fig 4. Immunohistochemical detection of VEGF in ischemic penumbra tissue at day 7 after tMCAO. (A) Ad group, (B) Ad-HIF-1α group. Bar=50µm. (B) Quantitative analysis of HIF-1α-positive cells in ischemic penumbra tissue. (# p > 0.05, * p < 0.01).
avascular area and stimulates their proliferation in angiogenesis [26, 27]. Therefore, the induction of VEGF and other proangiogenic factors in the ischemic region leads to an increase in the vascular density and hence a decrease in the oxygen diffusion distance. Our study demonstrated that VEGF expression was increased by HIF-1α treatment. HIF-1α may activate the expression of additional genes that promote angiogenesis besides VEGF, such as the urokinase receptor, a receptor that can enhance cellular migration and invasion [28].

Neurogenesis and angiogenesis interact coordinately in the songbird higher vocal center. These results suggest that the microenvironment surrounding blood vessels, which is termed as a vascular niche, might play a pivotal role in neurogenesis in the adult brain [29]. In our study, the results demonstrated HIF-1α treatment promoted both neurogenesis and angiogenesis, and thus improved neurologic function following cerebral ischemia in rats. In conclusion, HIF-1α or its pathway may be a therapeutic target for ischemic stroke.

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**Conflicts of interest**

The authors declare there are no competing interests.

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