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Original Article

The HIF-1 inhibitor YC-1 decreases reactive astrocyte formation in a rodent ischemia model

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Abstract: Astrocytes become reactive after central nervous system injury, re-expressing glial fibrillary acidic protein (GFAP), vascular endothelial growth factor (VEGF), and nestin. Hypoxia-inducible transcription factor alpha (HIF-1α) is an important transcription factor for several genes including the VEGF and nestin genes, the expression of which generate reactive astrocytes and cause gliosis after cerebral ischemia. To evaluate the role of HIF-1α in reactive astrocyte formation, we applied the potent HIF-1α inhibitor YC-1 to a focal cerebral ischemia model and analyzed the expression of HIF-1α, VEGF, nestin, and GFAP. Quantitative real-time reverse transcription polymerase chain reaction and western blot analyses demonstrated that the expression of HIF-1α and its downstream genes (VEGF and nestin) were markedly attenuated in the YC-1-treated group versus the control group (HIF-1α, VEGF: $p < 0.01$; nestin: $p < 0.05$). GFAP expression was also effectively inhibited in the YC-1-treated group ($p < 0.05$). Immunohistochemical evaluations showed that GFAP-positive (GFAP+) cells in the YC-1-treated group were sparse in the peri-infarct area, while an immunofluorescence assay revealed that the number of VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes were decreased in the YC-1-treated group ($p < 0.05$). These results demonstrate that HIF-1α suppression decreases the formation of reactive astrocytes and gliosis that occur following focal ischemia.

Keywords: Astrogliosis, reactive astrocyte, HIF-1α, GFAP, VEGF, nestin, YC-1

Introduction

Astrocytes are the most abundant glial cell in the brain and provide important support for neuronal functions, for example by maintaining ion and water homeostasis, regulating neuronal synapses, supplementing neuronal glucose levels, and inducing tight junction formation between endothelial cells to reduce blood-brain barrier (BBB) permeability [1, 2]. Additionally, in the central nervous system (CNS), various pathologies including ischemia, trauma, and neurodegenerative disease, cause astrocytes to undergo characteristic morphological changes. These altered astrocytes, with hypertrophy of the cell body and processes, have been termed “reactive astrocytes” [3].

Reactive astrocytes appear in the peri-injured area, and their proliferation can be observed several days after injury [4]. The presence of reactive astrocytes is one of the most prominent changes observed after injury to the CNS. In addition to the morphological changes that occur in reactive astrocytes after CNS injury, reactive astrocytes show increased gene expression, including increased expression of glial fibrillary acidic protein (GFAP), nestin, and vascular endothelial growth factor (VEGF) [3, 5-9]. Reactive astrogliosis is also known to have both neuroprotective and neurotoxic effects after ischemia [10].

Under ischemic conditions, a cellular oxygen-sensing system is necessary in order to rapidly adapt to changes in oxygen tension. Hypoxia-inducible factor (HIF-1) is a transcription factor that modulates this oxygen-sensing molecular pathway. HIF-1 consists of α and β subunits, and has been widely studied in hypoxic or ischemic conditions, the results of which show that HIF-1 has both positive and negative roles [11-

13]. Interestingly, HIF-1α is required for activating hypoxia-inducible genes including VEGF and nestin [14-18], which together with HIF-1α can affect the formation of astrogliosis [19, 20]. Although the regulation of HIF-1α and reactive astrocyte formation is vital for protecting neurons and ensuring their survival after hypoxic/ischemic-type CNS injuries, few studies have examined the relationship between HIF-1α and astrogliosis [21].

YC-1 (3-(5-hydroxymethyl-2-furyl)-1-benzylindazole) is an HIF-1 inhibitor that degrades the C-terminal end of HIF-1α, resulting in little or no HIF-1α activity [22]. A previous study demonstrated suppression of HIF-1α by YC-1, showing changes in infarct volume and BBB permeability after YC-1 application in a rat model of ischemic stroke [23]. Here, we quantified the expression levels of HIF-1α and its downstream genes, VEGF and nestin, to confirm the inhibitory effect of YC-1 on HIF-1α. The expression of GFAP was also analyzed to identify the effects of HIF-1α on reactive astrocyte formation and gliosis after ischemia in a photothrombotic rat stroke model.

Materials and methods

Photothrombotic ischemia model

All surgical procedures and postoperative care were performed in accordance with the guidelines of the Chonnam National University Animal Care and Use Committee. Male Sprague-Dawley (SD) rats (200-250 g) were maintained on a 12-h light/dark cycle and allowed free access to food and water.

A focal cerebral cortical lesion was induced in these animals by photothrombosis [24]. Briefly, each rat was anesthetized with 5% isoflurane and maintained with 2% isoflurane in an oxygen/air mixture using a gas anesthesia mask in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Once deep anesthesia was verified, the rat was placed in a prone position on a heated surgical pad (regulated by a rectal probe) so that its body temperature could be maintained at 37 ± 0.5°C throughout the surgery. A 4.5-mm fiber-optic bundle from a cold light source was positioned onto the exposed skull over the left sensorimotor cortex (0.5 mm anterior to bregma and 3.7 mm lateral to the midline). After exposing the brain, 50 mg/kg of Rose Bengal (RB; Sigma) in normal saline was infused for 1 min into the left femoral vein via a microinjection pump. Then, the exposed brain was illuminated for 10 min by the cold light source. The scalp was then sutured and the rats were returned to their individual cages; all rats were kept warm under an infrared lamp until they fully recovered from the anesthesia. Animals for the sham control group received illumination after infusion of normal saline instead of RB.

YC-1 application

Twenty-four male SD rats were randomly divided into two groups. One group (control group, n = 12) was treated with only the photothrombosis. The other group (n = 12) was pretreated with YC-1 (Cayman Chemical Company, Ann Arbor, MI, USA) using methods modified from Yan et al. [23], in addition to photothrombosis surgery. Briefly, a solution of YC-1 mixed with 1% dimethyl sulfoxide (DMSO) at a dose of 2 mg/kg body weight was injected through the femoral vein at 24 h and 30 min before the onset of ischemia. The same volume of the DMSO vehicle solution was injected into rats in the control group. Three rats from each group were evaluated immediately after and 1, 3, and 7 days after ischemia.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay

RNA was obtained from the ischemic cerebral cortex using TRIzol™ (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using Taqman RT reagents (Applied Biosystems), following the manufacturer's instructions. Real-time qRT-PCR was carried out on 25 ng equivalents in triplicate on an Applied Biosystems (AB) 7300 Sequence Detection System, using SYBR Green Assays for HIF-1α, VEGF-A, nestin, and GFAP (Life Technologies, Grand Island, NY, USA). Threshold amplification cycle-number data from multiple plates were combined using AB Relative Quantitation software (SDS1.2) and the delta-delta Ct method. All primers used in this study are detailed in **Table 1**.

Western blots

To analyze the protein expression patterns in the ischemic hemisphere, protein extracts were prepared from the tissues. First, the injured

Table 1. Primers used in the qRT-PCR analyses

Gene	Sense	Anti-sense
HIF-1α	TCAAGTCAGCAACGTGGAAG	TATCGAGGCTGTGTCGACTG
VEGF	CACATAGGAGAGATGAGCTTC	CCGCCTCGGCTTGTCACAT
Nestin	AGAGAAGCGCTGGAACAGAG	TTCCAGGATCTGAGCGATCT
GFAP	GAAGAAAACCGCATCACCAT	GCACACCTCACATCACATCC
GAPDH	GGGCATCCTGGGCTACACTGA	CCTTGCTGGGCTGGGTGGT

ischemic hemisphere was removed 3 days after surgery and the samples were frozen. PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Korea) was added to 1.5-mL tubes containing the frozen samples according to the manufacturer's protocol. The tissue samples were cut into small pieces on ice using microscissors for 30 min. The samples were centrifuged at 14,000 × *g* and the protein-containing supernatant was then transferred into a new tube. The protein concentration was measured using the bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL, USA).

The protein expression of HIF-1α, VEGF, nestin, and GFAP were investigated. Samples containing 50 μg of protein were separated by sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (PALL, Port Washington, NY, USA). After the transfer, membranes were incubated for 1 h at room temperature in blocking buffer (5% non-fat dry milk in Tris-buffered saline with 0.2% Tween-20), and then probed overnight at 4°C with one of the following primary antibodies: HIF-1α (1:500; Novus Biologicals, Littleton, CO, USA), VEGF (1:1,000; Santa Cruz, Santa Cruz, CA, USA), nestin (1:1,000; Millipore), GFAP (1:1000; Millipore), β-actin (1:25,000; Sigma-Aldrich), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:80,000: Cell signaling). The reaction was developed using luminol/peroxide chemiluminescence (Millipore). Optical densities were measured on a Fujifilm LAS-3000 luminescent image analyzer (Fuji Photo Film, Tokyo, Japan).

Immunohistochemistry and immunofluorescence assay

The brains were removed from the animals, with or without YC-1 (n = 3 for each group), 3 days after ischemic injury. After standard histological processing, hematoxylin and eosin-stained slides of the brain specimens were reviewed to determine the infarct area. Tissue blocks were then selected from the infarct area for formalin fixation and paraffin embedding of the brain tissue.

Immunostaining was performed using the standard avidin-biotin complex (ABC) method. Briefly, representative paraffin blocks were cut into consecutive 4-μm thick sections, and immunohistochemical staining was carried out using the Microprobe Immuno/DNA stainer (Fisher Scientific, Pittsburgh, PA, USA). Sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. The sections were then subjected to pressure cooker heat in 10 mM citrate-phosphate buffer (pH 6.0) for antigen retrieval. Sections were incubated in the following antibodies at 4°C overnight: HIF-1α (1:100; Sigma), VEGF (1:200; Santa Cruz Biotech), nestin (1:100; Millipore), and/or GFAP (1:1,000; Millipore). The streptavidin-horseradish peroxidase (Research Genetics, USA) detection system was applied to the capillary channels, followed by incubation at 37°C for 1 h. After drainage, the tissue sections were ready for chromogen reaction with 0.02% diaminobenzidine. The sections were counterstained with hematoxylin and mounted in Universal Mount (Research Genetics). For double immunofluorescence staining, Alexa Fluor® 488 (Invitrogen) and Cy3® (Invitrogen), with 4',6-diamidino-2-phenylindole as a counterstain, were conjugated with the appropriate secondary antibody for detecting the primary antibodies. Sections were incubated for 60 min at room temperature in the secondary antibody-fluorophore conjugates.

The total number of VEGF and nestin immunopositive reactive astrocytes within a defined grid area was counted, based on methods modified from Magnoni et al. [25]. Each image was captured by a KY-F70B digital camera system (JVC, Tokyo, Japan) mounted on a Nikon E600 microscope (Nikon, Tokyo, Japan), using × 200 magnification. Each × 200 magnification area was 0.48 mm^2.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0 software for Windows (SPSS, Chicago, IL, USA). Data are expressed

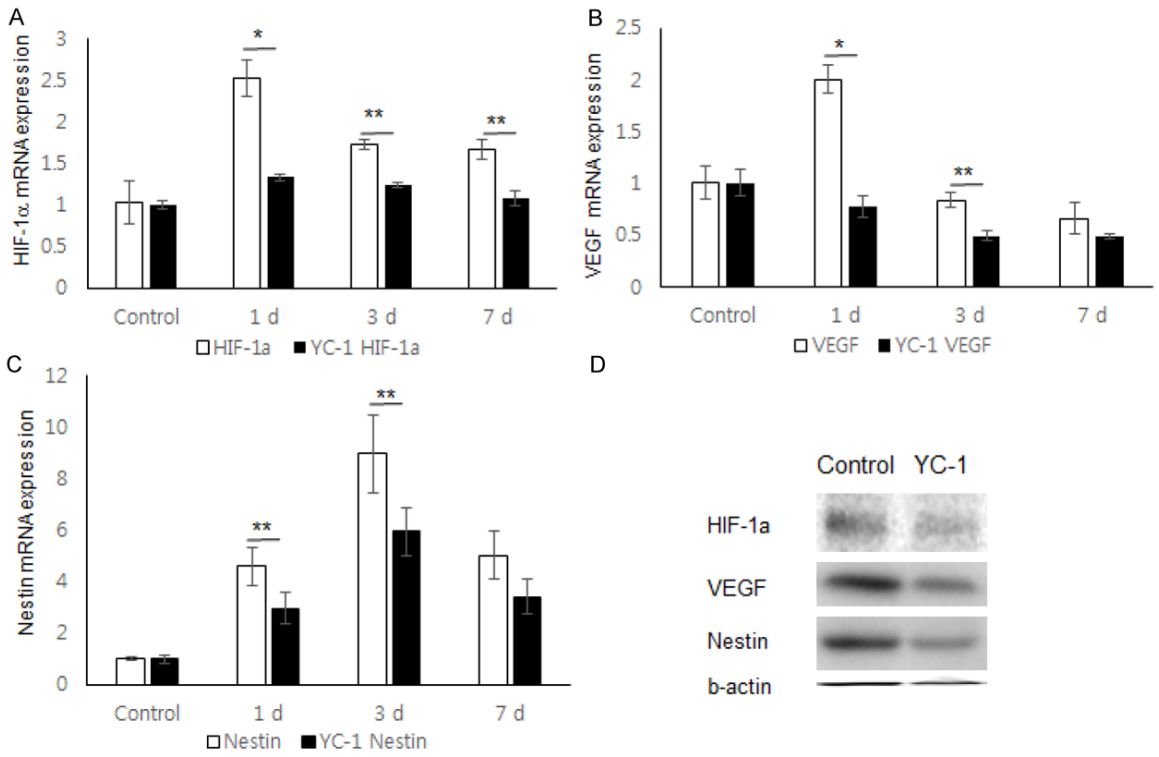

Figure 1. Suppression of HIF-1α, VEGF, and nestin by YC-1. The expression of HIF-1α and its downstream genes, namely VEGF and nestin, were analyzed by real-time qRT-PCR and western blotting. A-C. Results of the qRT-PCR analysis showing that YC-1 effectively inhibited the expression of HIF-1α, VEGF, and nestin. *$p < 0.01$, **$p < 0.05$. D. Western blots of protein samples taken 3 days after stroke demonstrated suppression of the HIF-1α, VEGF, and nestin protein levels by YC-1.

as the mean ± the standard deviation. Analyses were performed using Student's *t*-tests (for comparisons between two groups) or analyses of variance (ANOVAs) followed by Bonferroni *post-hoc* tests (for comparisons among multiple groups). The criterion for statistical significance was $p < 0.05$. All measurements were analyzed by observers blinded to the treatment groups.

Results

The expression of HIF-1α, VEGF, and nestin in a focal stroke rat model and inhibition by YC-1

Analysis of HIF-1α mRNA expression using qRT-PCR showed that the level of HIF-1α increased rapidly and peaked at 1 day after ischemia in the control group. However, in the group pretreated with YC-1, the HIF-1α mRNA level did not significantly increase after stroke (**Figure 1A**). The greatest significant ($p < 0.01$) difference in HIF-1α mRNA levels between the control and YC-1 groups was observed at 1 day after stroke, however significant gaps between the groups were also identified on days 3 and 7 after stroke ($p < 0.05$). As shown in **Figure 1B** and **1C**, the expression of downstream genes regulated by HIF-1α, namely VEGF and nestin, was effectively suppressed by YC-1. In the case of VEGF (**Figure 1B**), the mRNA levels between the two groups showed statistically significant differences at 1 and 3 days after stroke ($p < 0.01$ and $p < 0.05$, respectively). Compared with the control group, the group pretreated with YC-1 showed suppressed nestin mRNA levels at 1 and 3 days after stroke (**Figure 1C**, $p < 0.05$). Western blot analyses performed 3 days after ischemia demonstrated that YC-1 had effectively inhibited the expression of the HIF-1α, VEGF, and nestin proteins (**Figure 1D**).

Effects of YC-1 on reactive astrocyte formation in the peri-infarct area

To quantify HIF-1α activity during reactive astrocyte formation after focal ischemia, GFAP expression was analyzed by qRT-PCR and western blot assays (**Figure 2**). The GFAP mRNA level in the control group peaked at 3 days after

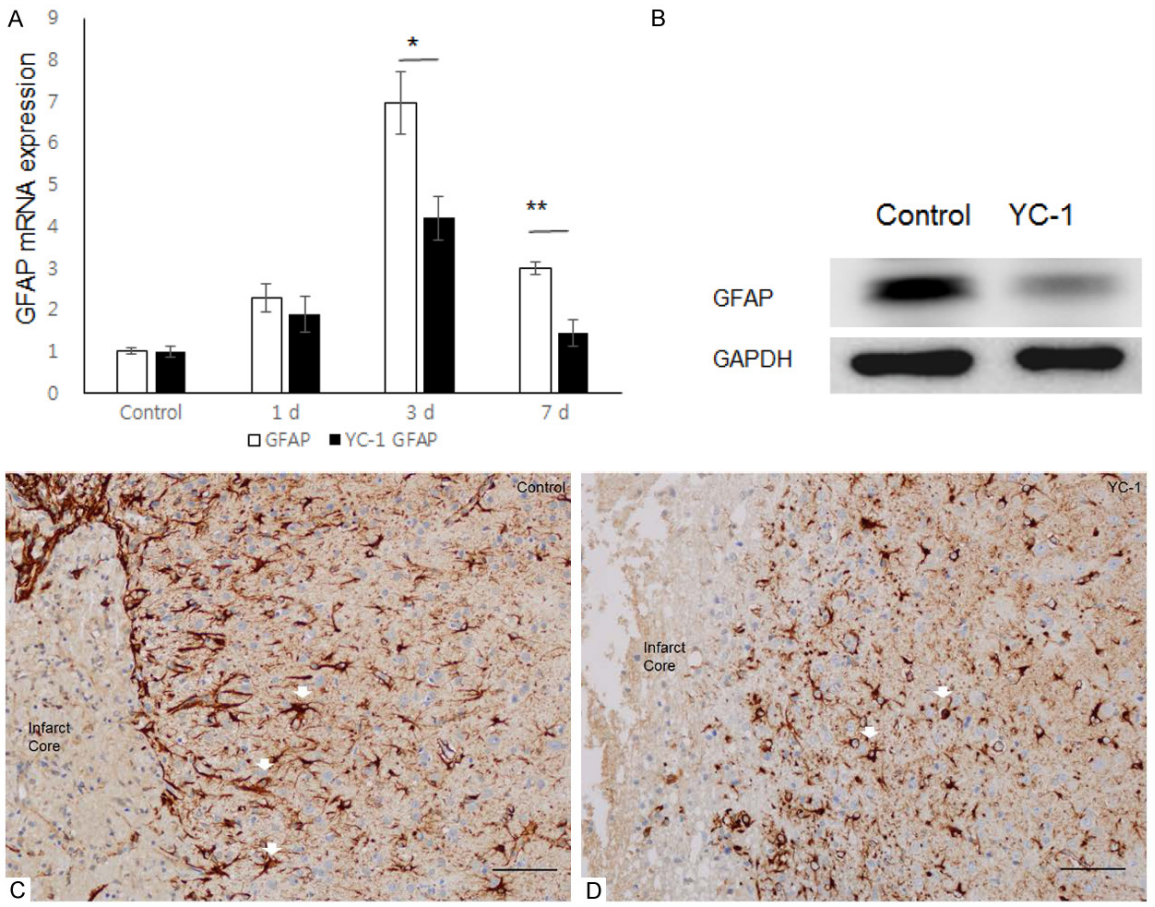

Figure 2. Suppression of GFAP by YC-1. The expression of GFAP was analyzed by qRT-PCR and western blotting. (A) Results of the qRT-PCR analysis demonstrating the inhibitory effect of YC-1 on the expression of GFAP (*$p < 0.01$, **$p < 0.05$). (B) Western blots of protein samples taken 3 days after stroke showing suppression of GFAP by YC-1. The immunoreactivity of GFAP in the reactive astrocyte (white arrow) is decreased in the YC-1 group (D) compared with the control group (C) 3 days after ischemia (scale bar = 100 μm).

stroke. In addition, the greatest difference between the control and YC-1 groups occurred at the same time (**Figure 2A**, $p < 0.01$). The western blots showed that the GFAP protein levels in the YC-1 group at 3 days after stroke were clearly suppressed compared with the levels in the control group (**Figure 2B**). In the control group, numerous reactive astrocyte-like cells with strong GFAP immunoreactivity were found in the peri-infarct area (**Figure 2C**). The number of GFAP-positive (GFAP+) cells, as well as the size of the infarct area, were reduced in the YC-1 group compared with the control group (**Figure 2D**).

Immunohistochemistry and immunofluorescence for GFAP, VEGF, and nestin in reactive astrocytes

The numbers of co-labeled VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes were counted at 3 days after stroke in five consecutive high-power fields (**Figure 3**). We found that the numbers of VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes were decreased in the group pretreated with YC-1 (**Figure 3B** and **3D**, respectively) relative to the control group (**Figure 3A** and **3C**, respectively), indicating that YC-1 significantly inhibited the expression of VEGF and nestin in reactive astrocytes ($p < 0.05$, **Figure 3E**).

Discussion

This study demonstrated that the expression and activity of HIF-1α were effectively inhibited by YC-1, with the greatest effect occurring 1 day after stroke. The inhibitory effect was related to the ability of YC-1 to suppress the rapid increase and peak in HIF-1α mRNA expression at 1 day post-stroke, as this increase was observed in the control group, but not in the

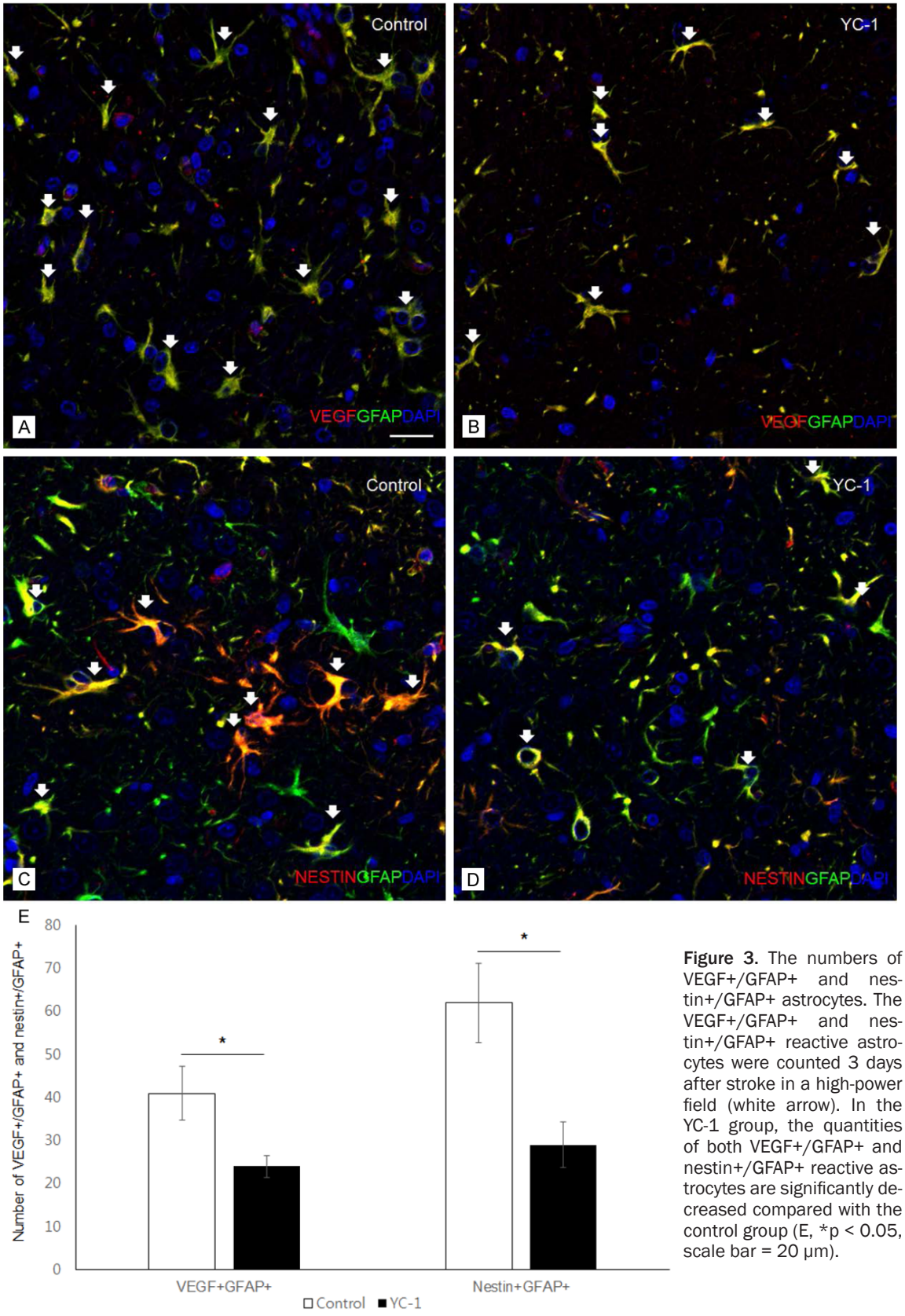

Figure 3. The numbers of VEGF+/GFAP+ and nestin+/GFAP+ astrocytes. The VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes were counted 3 days after stroke in a high-power field (white arrow). In the YC-1 group, the quantities of both VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes are significantly decreased compared with the control group (E, *$p < 0.05$, scale bar = 20 μm).

YC-1 group. The expression of VEGF mRNA, which is transcriptionally regulated by HIF-1, also reached its highest level at 1 day after ischemia. Induction of the HIF-1α-VEGF pathway under ischemic conditions has been established previously. After dimerization of HIF-1α and HIF-1β, HIF-1 binds to its binding site in the VEGF enhancer region and regulates transcriptional activation [26].

While the expression of HIF-1α and VEGF peaked at 1 day after stroke, the expression of nestin peaked at 3 days after stoke. Many previous studies have demonstrated a relationship between HIF-1α and nestin, thus establishing an HIF-1α-nestin pathway. Shih and Holland [18] showed that Notch activation enhanced nestin expression in gliomas, linking Notch to nestin. Notch activation as a reaction to hypoxia invoked other factors in the Notch signaling system, and HIF-1α was found to activate the transcription of genes downstream from notch by interacting with a stabilized intercellular domain of Notch [17]. This suggests that activation of the HIF-1α-Notch axis by hypoxic conditions increases the level of nestin in reactive astrocytes. However, the activation time of the HIF-1α-Notch-nestin axis was insufficient to explain the time difference between the peaks in HIF-1α and nestin mRNA expression.

The qRT-PCR and western blot data for VEGF and nestin show that while YC-1 did suppress the two genes effectively, this suppression was not complete in reactive astrocytes. These data indicate that there are other pathways regulating VEGF and nestin. Several growth factors such as epidermal growth factor (EGF), transforming growth factor alpha, and platelet-derived growth factor can upregulate VEGF mRNA expression without utilizing the HIF-1α-VEGF pathway [27]. In addition, one previous study showed that VEGF was elevated in HIF-1α-knockout reactive astrocytes under mild hypoxic conditions, suggesting that an HIF-1α-independent VEGF regulatory pathway must be present to explain the reactive astrocyte formation [28]. Nestin expression in reactive astrocytes was found to be induced and regulated by the extracellular signaling molecule, EGF, through the Ras-Raf-extracellular signal-regulated kinase pathway [29]. These previous studies showed that various complex molecular pathways influence the expression of VEGF and nestin in reactive astrocytes, however further studies on the activation of such alternative pathways under ischemic conditions are necessary to fully understand the relationships between HIF-1α, VEGF, and nestin.

In addition to nestin, GFAP mRNA expression also peaked at 3 days after ischemia. This was consistent with the time frame within which reactive astrocytes initiate their hypertrophic and proliferative changes [4]. The exact molecular relationship between HIF-1α and GFAP has not yet been determined for hypoxic brain tissue. In this study, inhibition of HIF-1α by YC-1 suppressed GFAP expression (mRNA and protein), indicating that HIF-1α helps regulate GFAP expression in reactive astrocytes. Indeed, in a mouse model of oxygen-induced retinopathy, YC-1 treatment suppressed the hypoxia-induced overexpression of GFAP in Müller and R28 cells [30].

Upon reviewing our immunohistochemical data, we found that pretreatment with YC-1 reduced not only the expression of VEGF and nestin, but also the size of the infarct area. In addition, the potential therapeutic effect of YC-1 could be assessed from the point of view of regulating reactive astrocyte formation through suppression of HIF-1α. However, the therapeutic potential of YC-1 is still debatable. One previous study demonstrated that the suppression of HIF-1α by YC-1 could inhibit the increased BBB permeability caused by ischemia [13]. On the other hand, another study found that YC-1 exaggerated the infarct volume and increased the mortality rate despite ameliorating the disturbances in BBB permeability [23]. The results of this latter study are different from those reported herein, suggesting that further studies are needed to determine whether YC-1 could be therapeutically useful for patients with cerebral ischemic injury.

Our immunofluorescence analyses demonstrated that the group pretreated with YC-1 had decreased numbers of VEGF+/GFAP+ and nestin+/GFAP+ reactive astrocytes in the peri-infarct area. VEGF, nestin, and GFAP all affect the reactive astrogliosis observed in the brain following ischemia. VEGF alters astrogliosis by increasing astrocyte proliferation and promoting the expression of growth factors through autocrine signaling [19]. In an experiment using mice with a photolytic injury, nestin+ reactive astrocytes exacerbated glial scar formation by

extending processes, many of which oriented radially outwards from the lesion core [20].

The effects of reactive astrocytes and astrogliosis following ischemic brain injuries are gradually being revealed through extensive studies and the use of several different animal models. These studies have demonstrated that reactive astrocytes are mainly found in the peri-infarct area. Furthermore, after CNS injury, migration of reactive astrocytes toward the peri-infarct area, which is mediated by the phosphorylation of STAT3, separates the lesion from the surrounding unaffected area, isolating the unaffected area from the toxic environment of the lesion and improving the recovery process [31-34]. It was also noted that reactive astrocytes attenuated acute neurodegeneration and improved BBB repair [35-37]. In addition, downregulation of aquaporin-4 in reactive astrocytes contributes to the reduction of post-ischemic edema [38], and reactive oxygen species and glutamate are removed from the para-ischemic area by reactive astrocytes [39, 40]. While these positive effects occur mainly during the acute stage after injury, the negative effects of reactive astrogliosis on neural regeneration appear at later stages of CNS injury [41-44]. Additional studies are needed to identify exactly when the beneficial effects end and detrimental effects begin, since only at that point would downregulation or termination of reactive gliosis optimally protect neurons and minimize brain damage in ischemic conditions.

In this study, the inhibitory effects of YC-1 on HIF-1α, VEGF, and nestin were confirmed, and we newly identified its inhibitory effects on GFAP expression and reactive astrocyte formation. Although some HIF-1α-independent pathways exist and appear to moderately influence the suppression of hypoxia-induced genes and reactive astrocyte formation, YC-1 was found to suppress these genes effectively in ischemic brain tissue. Thus, HIF-1α is a major regulatory gene that controls multiple downstream genes associated with the formation of astrogliosis, including VEGF, nestin, and GFAP. As described above, HIF-1α and reactive astrogliosis have both positive and negative effects on the protection and survival of neurons after stroke. The effective suppression of HIF-1α and reactive astrocyte formation by YC-1 could lead to the development of improved treatments and prognoses for patients with stroke.

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Disclosure of conflict of interest

None.

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