

# Hypoxia Inducible Factor 1 $\alpha$ Plays a Key Role in Remote Ischemic Preconditioning Against Stroke by Modulating Inflammatory Responses in Rats

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**Background**—Limb remote ischemic preconditioning (RIPC) protects against brain injury induced by stroke, but the underlying protective mechanisms remain unknown. As hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is neuroprotective in stroke and mediates neuroinflammation, we tested the hypothesis that HIF-1 $\alpha$  is a key factor of RIPC against stroke by mediating inflammation.

**Methods and Results**—Stroke was induced by transient middle cerebral artery occlusion in rats, and RIPC was conducted in both hind limbs. The HIF-1 $\alpha$  mRNA was examined by quantitative reverse transcription polymerase chain reaction after RIPC. In addition, inflammatory cytokines in the peripheral blood and brain were measured using the AimPlex multiplex immunoassays. Data showed that RIPC reduced the infarct size, improved neurological functions, and increased HIF-1 $\alpha$  mRNA levels, interleukin (IL)-4, and IL-10 protein levels in the peripheral blood. Intraperitoneal injection of the HIF activator, dimethylxaloylglycine, reduced the infarct size and inhibited interferon- $\gamma$  protein levels, while promoting IL-4 and IL-10 protein levels, while decreasing interferon- $\gamma$  protein levels in both the peripheral blood and ischemic brain. In addition, injection of dimethylxaloylglycine had a synergistic effect with RIPC on reducing infarction and improving neurological functions, as well as decreasing interferon- $\gamma$  in the peripheral blood and ischemic brain. In contrast, injection of the HIF inhibitor, acriflavine hydrochloride, abolished the protective effects of RIPC on infarction, and reduced IL-4 and IL-10 protein levels in both the peripheral blood and ischemic brain.

**Conclusions**—We conclude that HIF-1 $\alpha$  plays a key role in RIPC, likely mediated by a systemic modulation of the inflammatory response. (*J Am Heart Assoc.* 2018;7:e007589. DOI: 10.1161/JAHA.117.007589.)

**Key Words:** cerebral ischemia • hypoxia inducible factor-1 $\alpha$  • inflammatory cytokines • remote ischemic preconditioning • reperfusion

Ischemic stroke is the most common acute cerebrovascular disease worldwide. Thousands of clinical trials of pharmacological agents in acute stroke have failed to achieve expected outcomes.<sup>1</sup> Therefore, nonpharmacological treatments, such as remote ischemic preconditioning (RIPC), have

become a hot research topic for stroke treatment.<sup>2–4</sup> Laboratory experiments have shown the effectiveness of RIPC in reducing brain infarction,<sup>5,6</sup> and pilot clinical studies have confirmed that RIPC is feasible and effective for stroke patients.<sup>7,8</sup> Despite extensive studies, the underlying protective mechanisms of RIPC against stroke remain elusive.

Hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is one of the most important transcriptional factors implicated in the hypoxic or ischemic brain. With normal oxygen levels, HIF-1 $\alpha$  is hydroxylated and polyubiquitinated for proteasomal degradation by prolyl hydroxylase (PHD).<sup>9</sup> However, HIF-1 $\alpha$  is accumulated in hypoxic and ischemic cells as PHD is inactivated.<sup>10</sup> HIF-1 $\alpha$  targets several critical cell signaling proteins and enzymes, including inducible nitric oxide synthase, vascular endothelial growth factor (VEGF), erythropoietin, glucose transporter 1, and glycolytic enzymes.<sup>10–12</sup> Accumulating evidence indicates that HIF-1 $\alpha$  plays key roles in neuroprotection against ischemic brain injury and spontaneous functional recovery from traumatic brain injury.<sup>9,13,14</sup> In addition, HIF-1 $\alpha$  contributes to the protective effect of RIPC against myocardial

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## Clinical Perspective

### What Is New?

- Remote ischemic preconditioning protects against brain injury by increasing hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) mRNA levels in the peripheral blood.
- Increases in HIF-1 $\alpha$  mRNA levels are associated with anti-inflammatory cytokines interleukin-4 and interleukin-10 protein levels in the peripheral blood.
- HIF inhibition abolishes the protective effect of remote ischemic preconditioning, while HIF promotion provides synergistic effects with remote ischemic preconditioning.

### What Are the Clinical Implications?

- Enhancing HIF activities may attenuate brain injury induced by stroke.
- HIF modulation may facilitate clinical translation of remote ischemic preconditioning for stroke treatment.

infarction.<sup>15,16</sup> Although the accumulation of HIF-1 $\alpha$  was found to be associated with the remote ischemic postconditioning that protects against cerebral ischemic injury,<sup>17</sup> there is no solid evidence that HIF-1 $\alpha$  is important for RIPC against stroke.

Neuroinflammation that occurs in the brain modulates brain injury after stroke.<sup>18,19</sup> In addition, systemic inflammation also contributes to brain injury.<sup>20</sup> Although HIF-1 $\alpha$  is implicated in inflammation, because it is stabilized by interleukin (IL)-1 $\beta$ , tumor necrosis factor- $\alpha$ , and transforming growth factor- $\beta$ ,<sup>10,21</sup> whether it modulates neuroinflammation and systemic inflammation in RIPC in stroke remains unknown.

In this study, we aim to provide insights into the roles of HIF-1 $\alpha$  in RIPC protection in stroke, and to explore whether it modulates inflammatory cytokines. First, we studied the effect of RIPC alone on HIF-1 $\alpha$  expression and protein levels of multiple inflammatory cytokines in the peripheral blood. Then, we used the HIF-1 $\alpha$  activator, dimethylxaloylglycine (DMOG), which promoted HIF-1 $\alpha$  activity by inhibiting the PHD enzyme, and the HIF-1 $\alpha$  inhibitor, acriflavine hydrochloride (ACF), to study the role of HIF-1 $\alpha$  in modulating the protective effects of RIPC against stroke.

## Materials and Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. The data are available from the authors upon request.

Male Sprague-Dawley rats weighing 280 to 320 g, purchased from Vital River Laboratory Animal Technology Co, Ltd (Beijing, China) were housed at a controlled room temperature

of 24°C, under a 12-hour light/dark cycle, had free access to food and water, and were randomly distributed in different groups. The number of animals in each group was 6 to 10. All procedures in this study were conducted according to the guidelines established by the University Animal Care and Use Committee of Capital Medical University.

## Middle Cerebral Artery Occlusion

Rats were anesthetized with 3% to 5% isoflurane in 70% nitrous oxide and 30% oxygen, and maintained with 1% to 3% isoflurane. For the middle cerebral artery occlusion (MCAo) model, ischemia and reperfusion were established as previously described.<sup>22,23</sup> During surgery, the common carotid artery, right internal carotid artery, and external carotid artery were exposed. A silica gel-coated nylon suture with a diameter of 0.38 $\pm$ 0.02 mm was inserted into the internal carotid artery; a length of 18 to 20 mm from the bifurcation of the external carotid artery was inserted to block the MCA, and the suture was withdrawn after 90 minutes of occlusion to allow the reopening of the MCA. Rat rectal temperature was maintained at 37 $\pm$ 0.5°C during the entire procedure. The rats in the sham group were subjected to the surgical procedure without MCAo.

## Remote Ischemic Preconditioning

Twenty-four hours before MCAo, RIPC was conducted in both hind limbs of rats anesthetized with 1% to 3% isoflurane. The 2 hind limbs were simultaneously tied with a strip gauze bandage to occlude blood circulation for 10 minutes, and then released for 10 minutes to allow reperfusion. The occlusion/reperfusion cycle was repeated 3 times.

## Drug Injections

To study the role of HIF-1 $\alpha$  in RIPC, both HIF inhibitor and activator were used. The HIF inhibitor, ACF (A8251; Sigma), was dissolved in PBS, adjusted to a concentration of 0.5 mmol/L with additional PBS, and stored at 4°C. The drug dosage was based on previous reports. To inhibit HIF-1 $\alpha$ , the ACF solution was IP injected at a dose of 1.5 mg/kg, 24 hours before stroke onset and every 24 hours thereafter until euthanized.<sup>9,14</sup>

The PHD inhibitor (HIF activator), DMOG (S7483; Selleck.cn), was dissolved in DMSO at a concentration of 5 mmol/L. For animal administration, the drug solution was further diluted in sterile saline and stored at -20°C. The frozen solution was brought to room temperature 10 minutes before injection, and a final dose of 8 mg/kg was IP injected 60 minutes after reperfusion in rats.<sup>9,11</sup> A control group of animals received an injection of the DMSO vehicle solution.

## Behavioral Tests

A battery of behavioral tests were conducted to evaluate the effects of RIPC and modulation of HIF on neurological functions after stroke, including the home cage, postural reflex, and tail hang test, as detailed in our previous studies.<sup>24,25</sup> All behavioral tests were performed by a researcher who was blinded to the experimental conditions. The rats were handled for 3 days before surgery, and then their baseline was tested the day before surgery. All behavioral tests were then conducted 48 hours after stroke onset.

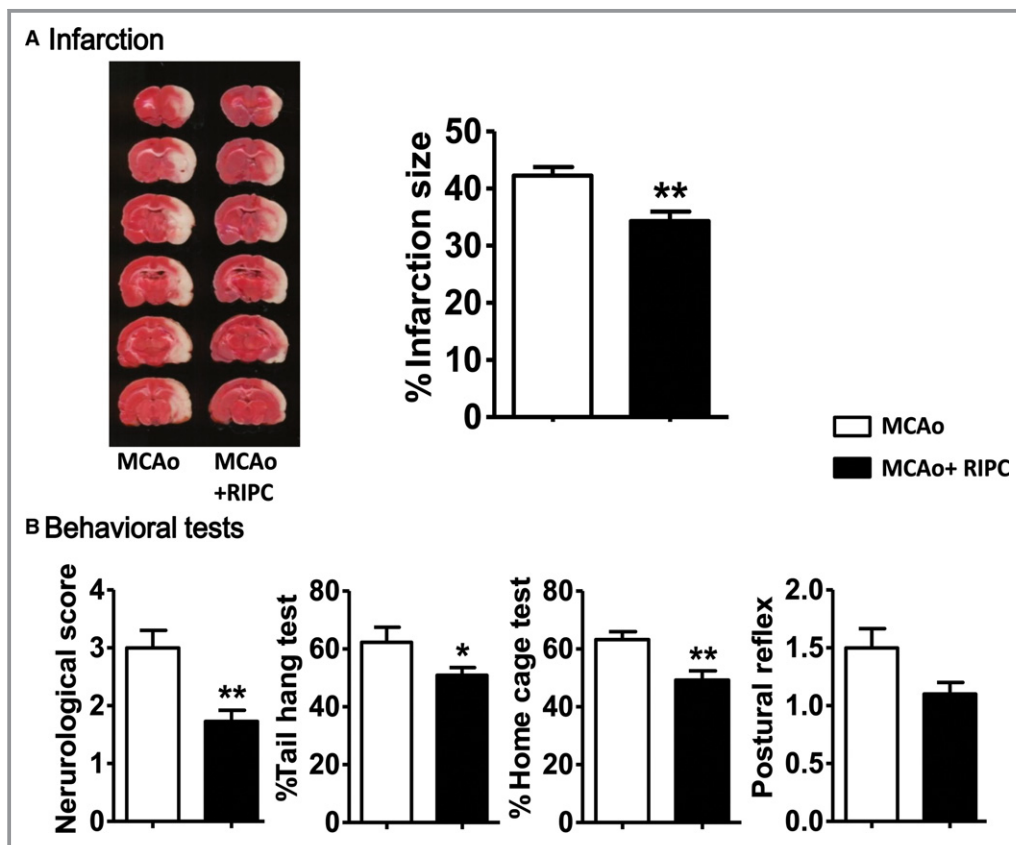
For the tail hang test, a head turn with more than 90° was counted when the rat tail was hung 10 to 20 cm above the floor. Each rat was hung 20 times, and the percentage of head turns was calculated. For the home cage limb test, the number of times the rat used its forelimbs to explore the cage wall was counted, including the ipsilateral, contralateral, or when both forelimbs touched the cage wall. These tests were conducted and recorded 20 times. Of the 20 recordings, the percentage of times the ipsilateral forelimb was used was calculated using this formula:  $(\text{ipsilateral} + [\text{both}/2]) \times 100\%$ .

For the postural reflex test, the rat was placed on a table, with its shoulder held with 1 hand and pushed 3 times for nearly 20 cm. A score of 0 was given if the rat gripped the table vigorously during the push, a score of 1 was give if the rat showed less resistance, and a score of 2 was given if the rat offered no resistance.

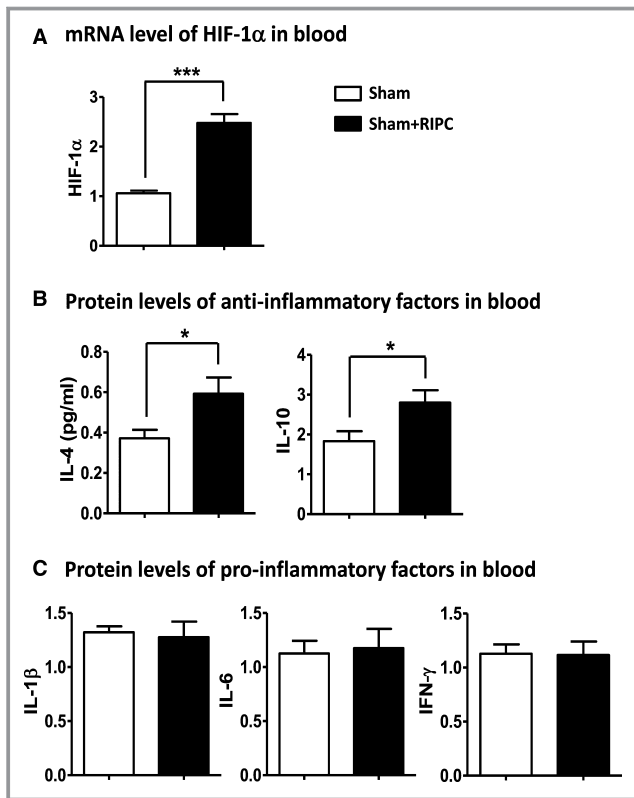
We also measured neurological deficits to evaluate stroke outcomes 48 hours after reperfusion using the Longa scoring system, in which the scores were based on the following criteria: 0=no deficit, 1=failure to extend left forepaw, 2=circling to the left, 3=falling to the left, 4=failure to walk spontaneously and loss of consciousness, and 5=death.<sup>26,27</sup>

## Infarct Size Measurement

The infarct size was measured 48 hours after reperfusion by 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining after the animals were euthanized. The nonischemic hemisphere and the nonischemic region in the ischemic hemisphere were



**Figure 1.** RIPC reduced infarct size and improved neurological function and the behavioral test after stroke. A, Representative images and infarct volume of TTC staining in the MCAo and MCAo+RIPC group. B, Neurological score and behavior test. Statistical analysis was performed by ANOVA. \*, \*\* vs MCAo,  $P < 0.05$ ,  $0.01$ , respectively. (N=8–10 per group). MCAo indicates middle cerebral artery occlusion; RIPC, remote ischemic preconditioning; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride.



**Figure 2.** The effects of RIPC on HIF-1 $\alpha$  expression and inflammatory cytokines in the peripheral blood. A, The mRNA level of HIF-1 $\alpha$  was assessed by real-time RT-PCR. B, The protein levels of anti-inflammatory cytokines include IL-4 and IL-10, respectively. C, The protein levels of pro-inflammatory cytokines, IL-1 $\beta$ , IL-6, and IFN- $\gamma$ , respectively. These cytokine levels were assessed using AimPlex multiplex immunoassays. (N=6–7 per group). Statistical analysis was performed using *t* test. \*, \*\*\* vs sham,  $P<0.05$ , 0.001, respectively. HIF-1 $\alpha$  indicates hypoxia inducible factor 1 $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; RIPC, remote ischemic preconditioning; RT-PCR, reverse transcription polymerase chain reaction.

measured, from which the infarct was calculated according to the following formula:  $([\text{area of the nonischemic hemisphere} - \text{area of the nonischemic region in the ischemic hemisphere}] / \text{area of the nonischemic hemisphere}) \times 100\%$ . The detailed protocol has been described previously.<sup>28</sup>

### Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

To measure HIF-1 $\alpha$  mRNA expression, total RNA was isolated from blood collected from the heart of the animals before they were euthanized. The cortical penumbra was collected on ice and stored at  $-80^{\circ}\text{C}$  immediately after the animals were euthanized. RNA was extracted with TRIzol reagent (Cat# 15596-026; Life Technologies, CA) according to the manufacturer's instructions. The purified RNA was then reverse-

transcribed into cDNA using the Reverse Transcription System (Cat# E6300S; New England BioLabs<sup>®</sup> Inc, Ipswich, MA). Quantitative reverse transcription polymerase chain reaction analysis of the mRNA level of HIF-1 $\alpha$  (HIF-1 $\alpha$  F, TCATCCAAGGAGCCTTAACC; HIF-1 $\alpha$  R, AAGCGACATAGTAGGGGCAC) was performed using the SYBR Green Prime Script kit (RR420A, TAKARA). GAPDH (GAPDH F, GGCAAGTTCAACGGCACAG; GAPDH R, CGCCAGTAGAC TCCACGACAT) was chosen as the housekeeping gene. The real-time polymerase chain reaction program steps were as follows:  $95^{\circ}\text{C}$  for 5 minutes, 45 cycles at  $95^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 5 s, and  $72^{\circ}\text{C}$  for 10 s, followed by  $72^{\circ}\text{C}$  for 1 minute.

### AimPlex Multiplex Immunoassays

To study how RIPC affects inflammation, we measured 5 cytokines: IL-4, IL-10, IL-1 $\beta$ , IL-6 and interferon (IFN)- $\gamma$ , in plasma using the commercially available AimPlex rat inflammation assay kit (Beijing Quantobio, China). The procedure was conducted according to the manufacturer's instructions. Briefly, 45  $\mu\text{L}$  of capture bead working suspension and a 45- $\mu\text{L}$  sample were sequentially shaken 750 times per minute for 60 minutes in the dark, followed by 3 washings in 100  $\mu\text{L}$  washing buffer. The biotin-conjugated antibodies (25  $\mu\text{L}$ ) were added, and the wells were sealed and shaken for 30 minutes in the dark. After washing 3 times with washing buffer, streptavidin-PE was added, and shaken for 20 minutes in the dark. Finally, 100 to 200  $\mu\text{L}$  1x Reading buffer was added to each well, the fluorescence signals of the samples were acquired using a flow cytometer (NovoCyte, ACEA Biosciences, Inc), and the inflammatory factors were analyzed with FCAP Array 3.0 software.<sup>29</sup>

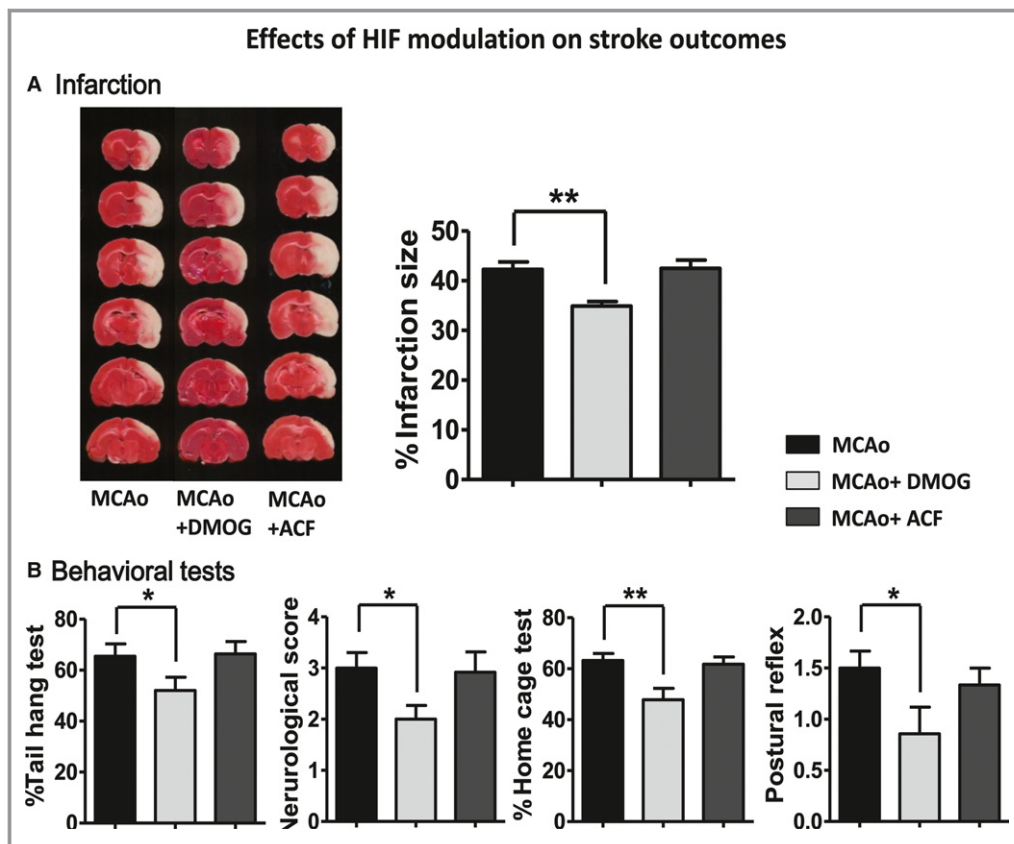
### Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad software, Inc, La Jolla, CA). Values were represented as means $\pm$ SEM. Differences between experimental groups were analyzed by Student *t* test or 1-way ANOVA with Bonferroni test as a post hoc test. A value of  $P<0.05$  was considered statistically significant.

## Results

### RIPC Protects Against Stroke

First, we confirmed that RIPC was neuroprotective in our new rat RIPC model, in which RIPC was conducted by a noninvasive occlusion of hind limb blood flow using a gauge bandage, in contrast to the invasive, direct femoral artery occlusion in previous studies.<sup>5,6</sup> The TTC staining results showed that RIPC significantly reduced infarct sizes from  $42.52 \pm 1.51\%$  to



**Figure 3.** The effects of the HIF-1 $\alpha$  activator DMOG and antagonist ACF on stroke outcomes. A, Representative images and infarct volume of TTC staining in the MCAo, MCAo+DMOG, and MCAo+ACF groups. B, Neurological scores and behavioral tests including tail hang test, home cage limb test, and postural reflex test. (N=8–10 per group). Statistical analysis was performed by ANOVA. \*, \*\* vs MCAo,  $P<0.05$ ,  $0.01$ , respectively. ACF indicates acriflavine hydrochloride, HIF-1 $\alpha$  inhibitor; DMOG, dimethylxaloylglycine, HIF-1 $\alpha$  activator; HIF-1 $\alpha$ , hypoxia inducible factor 1 $\alpha$ ; MCAo, middle cerebral artery occlusion; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride.

$34.13 \pm 1.62\%$  (Figure 1A), attenuated neurological scores (Figure 1B), and improved behavioral performance in the tail hang test and home cage limb test (Figure 1B). Although there was no significant difference in the postural reflex test, RIPC showed a trend toward the promotion of functional recovery (Figure 1B).

### Effect of RIPC on HIF-1 $\alpha$ Expression and Inflammatory Factors in the Peripheral Blood

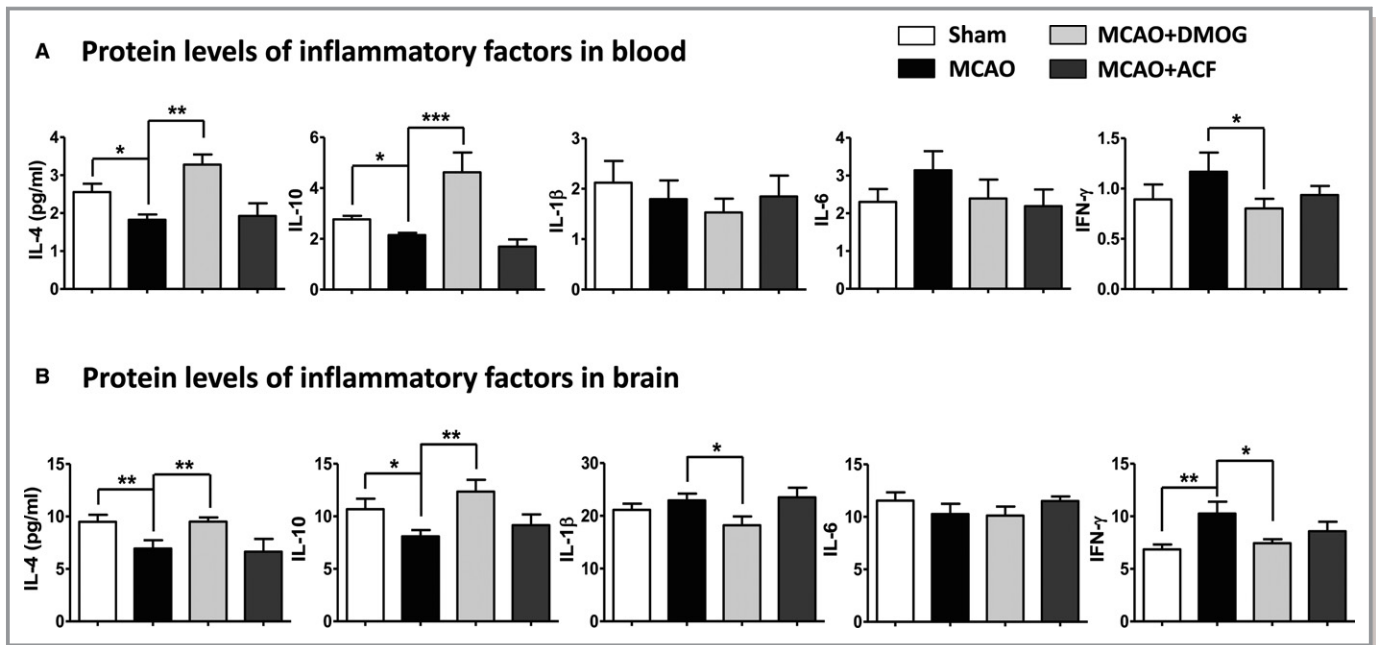
To test whether HIF-1 $\alpha$  is directly affected by RIPC, and whether the inflammatory status is altered by RIPC in the peripheral blood, we measured the effect of RIPC alone, without stroke, on HIF-1 $\alpha$  mRNA levels and protein levels of the anti-inflammatory cytokines, IL-4 and IL-10, and the pro-inflammatory cytokines, IL-1 $\beta$ , IL-6, and IFN- $\gamma$ , in the peripheral blood. The real-time PCR results showed that mRNA expression of HIF-1 $\alpha$  was upregulated by RIPC alone, and 24 hours after RIPC application

(Figure 2A). In addition, Aimplex multiplex immunoassays were used to detect inflammatory cytokines in the blood after RIPC. The results showed that RIPC increased IL-4 and IL-10 protein expression (Figure 2B) in comparison to the sham group. However, no significant changes in anti-inflammatory cytokines (Figure 2C) were observed.

### HIF-1 $\alpha$ Activation Attenuates While HIF-1 $\alpha$ Inhibition Does Not Alter Brain Injuries After Stroke

If HIF-1 $\alpha$  is important for brain protection, alterations of HIF-1 $\alpha$  activity will alter brain injuries induced by stroke. To promote HIF-1 $\alpha$  activity, the PHD inhibitor, DMOG, was injected 60 minutes after reperfusion; to inhibit HIF-1 $\alpha$  activity, the HIF-1 $\alpha$  inhibitor, ACF, was injected 24 hours before MCAo and repeated every 24 hours until the animals were euthanized. The results showed that the HIF-1 $\alpha$  agonist,





**Figure 4.** Effects of the HIF activator DMOG and inhibitor ACF on inflammatory cytokines in the blood and brain following MCAo. A, Protein levels of the anti-inflammatory cytokines IL-4 and IL-10 and pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and IFN- $\gamma$ , respectively, in the peripheral blood. A total of 4 groups were evaluated: Sham, MCAo, MCAo+DMOG, and MCAo+ACF. B, Protein levels of IL-4, IL-10, IL-1 $\beta$ , IL-6, and IFN- $\gamma$  in the ischemic brain. All samples were collected 48 hours after stroke. (N=6–7 per group). \*, \*\*, \*\*\* vs MCAo,  $P < 0.05$ , 0.01, 0.001, respectively. ACF indicates acriflavine hydrochloride; DMOG, dimethylxaloylglycine; HIF, hypoxia inducible factor; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; MCAo, middle cerebral artery occlusion.

DMOG, reduced the infarct size from  $42.52 \pm 1.51\%$  to  $34.81 \pm 0.68\%$ , which was comparable to the protective effect of RIPC (Figure 3A). In addition, DMOG also attenuated neurological scores (Figure 3B) and improved behavioral performance after stroke (Figure 3B).

### Effect of DMOG and ACF on Inflammatory Factors in the Periphery and Brain Region Following Stroke

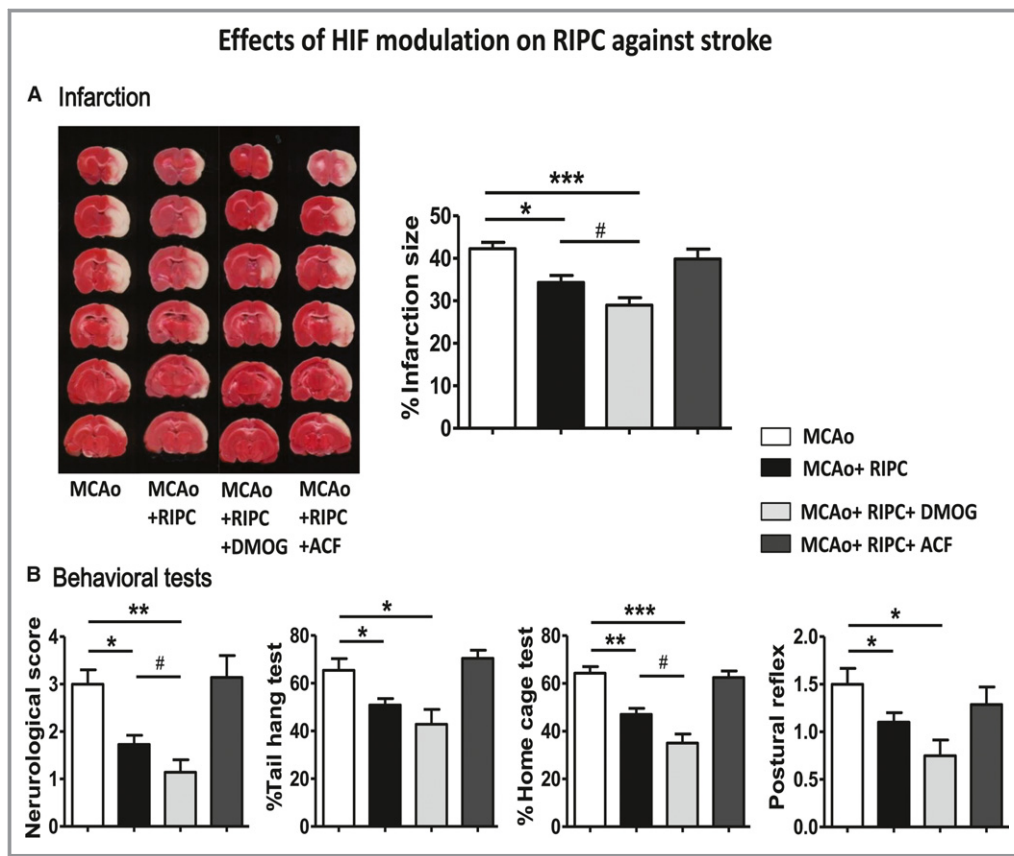
We further examined the effect of DMOG on inflammatory factors after stroke. Peripheral blood and penumbra tissue were collected from the heart and the cortex of the ischemic brain, and AimPlex multiplex immunoassays were used to detect multiple inflammatory cytokines in these samples. The results showed that DMOG significantly increased IL-4 and IL-10 levels in the peripheral blood (Figure 4A) and ischemic brain (Figure 4B) 48 hours poststroke while decreasing IL-1 $\beta$  and IFN- $\gamma$  levels in the peripheral blood and IFN- $\gamma$  levels in ischemic brain (Figure 4A and 4B). However, DMOG did not alter IL-1 $\beta$  levels in ischemic brain (Figure 4B) and IL-6 protein levels of in the blood (Figure 4A) or in the ischemic brain (Figure 4B) following stroke. Nevertheless, ACF injection did not affect any of the tested cytokines (Figure 4).

### Synergistic Effects of the HIF Activator DMOG With RIPC, and Antagonistic Effect of the HIF Inhibitor ACF With RIPC

After validating that the HIF activator DMOG inhibited while the inhibitor ACF did not alter brain injury induced by stroke, we further tested whether DMOG and ACF had synergistic and antagonistic effects with RIPC, respectively. The TTC staining results showed that DMOG further reduced infarct size in animals receiving RIPC (Figure 5A), suggesting that DMOG had a synergistic effect with RIPC. Such synergistic effects were also observed in the neurological score test (Figure 5B) and other behavioral tests, especially in the home cage test (Figure 5B). Conversely, the HIF-1 $\alpha$  inhibitor ACF abolished the protective effects of RIPC as measured by the infarct size, neurological score, and behavioral test (Figure 5).

### Effects of DMOG and ACF on Inflammatory Cytokines in the Peripheral Blood and Ischemic Brain of Animals Receiving RIPC

After examining the synergistic and antagonistic effects of the HIF activator and inhibitor with RIPC on brain injuries, we further assessed their effects on inflammatory factors. The

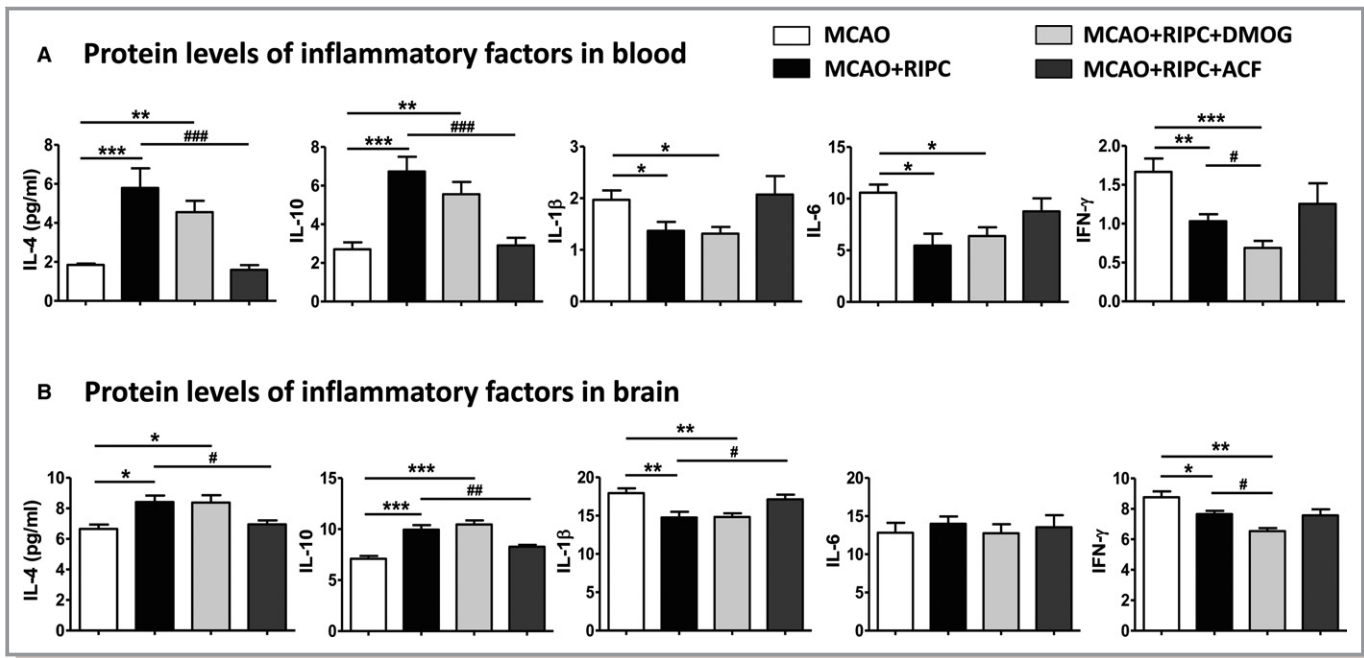


**Figure 5.** The effects of DMOG and ACF on stroke outcomes in stroke animals treated with RIPC. A, Representative images of TTC staining and the statistical results of infarct volume. B, Neurological score and behavioral tests of the tail hang test, home cage test, and postural reflex test. A total of 4 groups were assessed: MCAo, MCAo+RIPC, MCAo+RIPC+DMOG, and MCAo+RIPC+ACF group (N=8–10 per group). \*, \*\*, \*\*\* vs MCAo,  $P < 0.05$ , 0.01, 0.001, respectively. #  $P < 0.05$ , vs MCAo+RIPC. ACF indicates acriflavine hydrochloride; DMOG, dimethylxaloylglycine; HIF, hypoxia inducible factor; MCAo, middle cerebral artery occlusion; RIPC, remote ischemic preconditioning; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride.

results of the AimPlex multiplex immunoassays showed that in the peripheral blood, DMOG had no synergistic effects with RIPC on the reductions of IL-1 and IL-6 protein levels (Figure 6A), but it further reduced IFN- $\gamma$  protein levels (Figure 6A). Simultaneously, ACF had an antagonist effect on IL-1 $\beta$  but not on IL-6 and IFN- $\gamma$  protein levels (Figure 6A). In addition, unexpectedly, DMOG reduced IL-4 and IL-10 protein levels, and ACF reduced the levels even more in the peripheral blood compared with the RIPC group (Figure 6A). With respect to pro-inflammatory cytokines in the ischemic brain, DMOG had a synergistic effect with RIPC on IFN- $\gamma$  only (Figure 6B) but no synergistic effects on IL-1 $\beta$  and IL-6 (Figure 6B), while ACF did not alter their levels compared with RIPC (Figure 6B). Concerning anti-inflammatory cytokines in the ischemic brain, DMOG did not alter IL-4 and IL-10 protein levels compared with RIPC, but ACF reduced their levels (Figure 6B).

## Discussion

In the present study, we provide robust evidence that HIF-1 $\alpha$  is a critical factor contributing to the protective effect of RIPC against stroke, which is likely achieved by modulating neuroinflammation in the ischemic brain and systemic inflammation in the peripheral blood. First, we found that RIPC alone increased the expression of HIF-1 $\alpha$  mRNA and promoted protein levels of anti-inflammatory IL-4 and IL-10 in the peripheral blood. Second, the HIF-1 $\alpha$  activator, DMOG, generated protective effects similar to RIPC because it inhibited infarction and improved neurological functions. In addition, DMOG upregulated the anti-inflammatory cytokines IL-4 and IL-10, while inhibiting pro-inflammatory cytokine IFN- $\gamma$  in the peripheral blood and brain tissue. Third, the combination of RIPC with DMOG had a synergistic effect on the decrease in IFN- $\gamma$  protein levels in peripheral blood and



**Figure 6.** The effects of DMOG and ACF on protein levels of inflammatory cytokines in stroke animals receiving RIPC. A, Protein levels of the anti-inflammatory cytokines IL-4 and IL-10 and pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and IFN- $\gamma$  in the peripheral blood, respectively. A total of 4 groups were assessed: MCAo, DMOG+RIPC, MCAo+RIPC+DMOG, and MCAo+RIPC+ACF. B, Protein levels of IL-4, IL-10, IL-1 $\beta$ , IL-6, and IFN- $\gamma$  in the ischemic brain, respectively. (N=6–7 per group). \*, \*\*, \*\*\*, vs MCAo,  $P < 0.05$ , 0.01, 0.001, respectively. #, ##, ###, vs MCAo+RIPC,  $P < 0.05$ , 0.01, 0.001, respectively. ACF indicates acriflavine hydrochloride; DMOG, dimethylxaloylglycine; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; MCAo, middle cerebral artery occlusion; RIPC, remote ischemic preconditioning.

ischemic brain tissue. Fourth, in contrast to DMOG, the HIF-1 $\alpha$  inhibitor, ACF, abolished the protective effect of RIPC on infarction and neurological function. In addition, ACF inhibited the upregulation of anti-inflammatory factors induced by RIPC, including IL-4 and IL-10 in the peripheral blood and ischemic brain, while it inhibited the downregulation of proinflammatory factors induced by RIPC, including IL-1 $\beta$  in the ischemic brain. Collectively, these findings showed that HIF-1 $\alpha$  played a key role in the protective effect of RIPC by modulating inflammatory responses both in the brain and peripheral blood circulation.

HIF-1 $\alpha$  protein is a transcriptional factor that is degraded by the enzyme, PHD, within a few minutes under normal oxygen levels, but is stabilized and accumulated under hypoxic conditions.<sup>9,10</sup> This phenomenon results from the oxygen-dependent enzyme activity of PHD because PHD uses oxygen as a co-substrate. Once stabilized, HIF-1 $\alpha$  migrates to the nucleus and forms a heterodimer with the  $\beta$  subunit of the aryl hydrocarbon receptor nuclear translocator. The HIF-1 heterodimer binds hypoxia-response elements that promote the expression of >60 genes, including vascular endothelial growth factor, glucose transporter 1, and erythropoietin, under hypoxic conditions. The activities of HIF-1 $\alpha$  can be modulated by PHD inhibitors and drugs that interfere with heterodimer formation. In this study, we used the PHD inhibitor, DMOG, to promote and the heterodimer inhibitor, ACF, to inhibit HIF-1 $\alpha$  activity.

HIF-1 $\alpha$  has been shown to play important roles in hypoxic environments and ischemic diseases. Previous studies have demonstrated that overexpression of HIF-1 $\alpha$  ameliorates brain injury induced by stroke.<sup>9,14</sup> In addition, ischemic tolerance induced by hypoxic preconditioning resulted from the production of HIF-1 $\alpha$ ,<sup>16,30</sup> although it was controversial in another study.<sup>31</sup> The underlying protective mechanisms of HIF-1 $\alpha$  in ischemic stroke or myocardial infarction are probably a consequence of the ability of HIF-1 $\alpha$  to promote expressions of vascular endothelial growth factor, erythropoietin, and glucose transporter 1.<sup>32,33</sup> Furthermore, HIF-1 $\alpha$  is also an inflammation mediator because it is stabilized by interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ , and it plays key roles in leukocyte survival.<sup>10</sup>

To examine whether the protective role of HIF-1 $\alpha$  in RIPC is achieved by modulating inflammation, we examined the protein levels of both pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, and IFN- $\gamma$ , and the anti-inflammatory cytokines IL-4 and IL-10 in the peripheral blood and ischemic brain. We showed that DMOG increased IL-4 and IL-10 protein expression levels 48 hours after MCAo, and the combination of DMOG and RIPC further decreased IF- $\gamma$  protein levels, compared with RIPC in both the peripheral blood and ischemic brain. Conversely, ACF abolished the upregulation of IL-4 and IL-10 induced by RIPC in the blood and ischemic brain. Our results suggest that HIF-1 $\alpha$  improves stroke



outcomes, likely by upregulating IL-4 and IL-10 levels, and downregulating the IFN- $\gamma$  level. Therefore, because HIF-1 $\alpha$  is a critical factor for the protective effects of RIPC, RIPC may protect the brain by modulating systemic inflammation in the peripheral blood and neuroinflammation in the ischemic brain.

There are several limitations in this current study. First, cell-type-specific mRNA was not analyzed. It is necessary to identify the specific cell types that respond to RIPC via modulating HIF expression. In addition, mRNA levels do not equate to protein levels; thus, future studies should also examine the protein levels of HIF. Second, an interaction between brain injury and immune response was not addressed. Future works will address inflammatory cells and their functions in the ischemic brain after RIPC, and their correlation with brain injury and protection. Third, only a pharmacological inhibitor and an activator for HIF were used in this current study. As these agents are often nonspecific, genetically modified animal models should be used in the future. Fourth, only the acute protective effects of RIPC were tested. The long-term protective effects of RIPC should be examined in the future.

In summary, we provide strong evidence that RIPC promotes HIF-1 $\alpha$  activation in the peripheral blood, and that modulation of HIF-1 $\alpha$  activities not only affect ischemic brain injury, but also the protective effects of RIPC against stroke. We conclude that HIF-1 $\alpha$  contributes to the protective effect of RIPC, which may be achieved by modulating neuroinflammation in the ischemic brain and systemic inflammation in the peripheral blood circulation.

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## Disclosures

None.

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