Ischemic postconditioning enhances glycogen synthase kinase-3β expression and alleviates cerebral ischemia/reperfusion injury⋆

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Abstract

The present study established global brain ischemia using the four-vessel occlusion method. Following three rounds of reperfusion for 30 seconds, and occlusion for 10 seconds, followed by reperfusion for 48 hours, infarct area, the number of TUNEL-positive cells and Bcl-2 expression were significantly reduced. However, glycogen synthase kinase-3β activity, cortical Bax and caspase-3 expression significantly increased, similar to results following ischemic postconditioning. Our results indicated that ischemic postconditioning may enhance glycogen synthase kinase-3β activity, a downstream molecule of the phosphatase and tensin homolog deleted on chromosome 10/phosphatidylinositol 3-kinase/protein kinase B signaling pathway, which reduces caspase-3 expression to protect the brain against ischemic injury.

Key Words

cerebral ischemia/reperfusion; glycogen synthase kinase-3β; ischemic postconditioning; ischemic preconditioning; apoptosis; neural regeneration

Research Highlights

Ischemic postconditioning may enhance glycogen synthase kinase-3β activity, a downstream molecule of the phosphatase and tensin homolog deleted on chromosome 10/phosphatidylinositol 3-kinase/protein kinase B signaling pathway, which reduces caspase-3 expression, decreases cerebral infarct area and inhibits apoptotic cell death to protect the brain from ischemic injury.

Abbreviations

IPost, ischemic postconditioning; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; GSK-3β, glycogen synthase kinase-3β

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INTRODUCTION

Ischemia/reperfusion injury[1-3] is closely linked to loss of brain function in patients after a transient ischemic attack. Despite efforts to reduce cerebral ischemia/reperfusion injury, an ideal therapeutic approach for clinical neuroprotection against ischemia/reperfusion injury is still lacking. Since Zhao *et al* [4] first reported that myocardial ischemic postconditioning (IPost) can reduce myocardial ischemia/reperfusion injury in 2003, related studies have been performed. Research has shown that oxidative stress is a main factor of ischemia/reperfusion injury and that activation of the phosphatase and tensin homolog deleted on chromosome 10/phosphatidylinositol 3-kinase/protein kinase B (PTEN/PI3K/ AKT) can induce oxidative stress. However, most studies have focused on upstream events of the PTEN/PI3K/AKT signaling pathway, and few studies have

investigated the role of glycogen synthase kinase-3β (GSK-3β)[5-7], a downstream molecule. We hypothesize that IPost may attenuate ischemia/reperfusion injury and that GSK-3β may play a protective role. Therefore, the present study sought to verify whether GSK-3β was involved in IPost-mediated neuroprotection in a rat model of global brain ischemia.

RESULTS

Quantitative analysis of experimental animals
A total of 56 rats were selected, and 46 were used to establish models of global brain ischemia using the four-vessel occlusion method[8-9]. Of them, nine rats died during modeling and seven died during reperfusion. The remaining 30 model rats were randomly assigned to ischemia/reperfusion, IPost, and ischemic preconditioning groups. The IPost and ischemic preconditioning groups were subjected to brief reperfusion following and prior to 48 hours of reperfusion, respectively. The remaining 10 rats were used as the sham-surgery group.

IPost reduced cell death in the cerebral cortex
Following TUNEL, a large number of TUNEL-positive cells were observed in the cortex of rats subjected to ischemia/reperfusion injury, whereas TUNEL-positive cells were not detected in the cortex of sham-surgery group rats. The number of TUNEL-positive cells was significantly reduced in the cortex of the IPost and ischemic preconditioning groups compared with the ischemia/reperfusion group ($P < 0.01$; Figure 1, supplementary Figure 1 online).
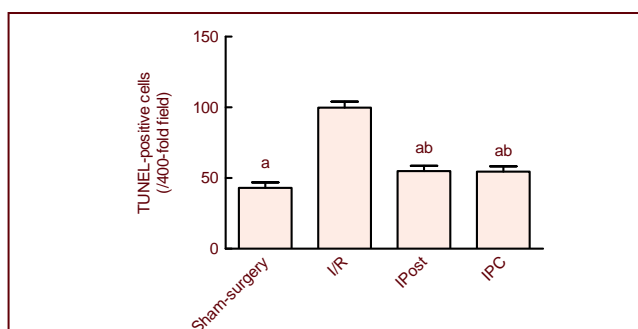

Figure 1   IPost reduced cell death following global brain ischemia.

Number of TUNEL-positive cells following global brain ischemia treated with IPost. Each bar indicates the mean ± SD of quadruplicate experiments. Different groups were compared by one-way analysis of variance and the Student-Newman-Keuls test. [a]$P <$ 0.01, *vs.* I/R group; [b]$P <$ 0.01, *vs.* sham-surgery group.

I/R: Ischemia/reperfusion; IPost: ischemic postconditioning; IPC: ischemic preconditioning

IPost reduced cerebral infarct area

Both IPost and ischemic preconditioning treatment significantly decreased cerebral infarct area 48 hours after reperfusion compared with the ischemia/reperfusion group ($P < 0.01$; Figure 2). Sham-surgery group rats were not injured.
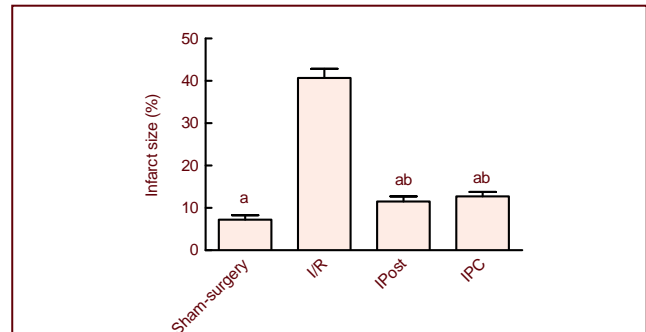

Figure 2   IPost reduced infarct size following global brain ischemia.

Each bar represents the mean ± SD of quadruplicate studies. [a]$P < 0.01$, *vs.* I/R group; [b]$P < 0.01$, *vs.* sham-surgery group. Different groups were compared by one-way analysis of variance and the Student-Newman-Keuls test.

I/R: Ischemia/reperfusion; IPost: ischemic postconditioning; IPC: ischemic preconditioning.

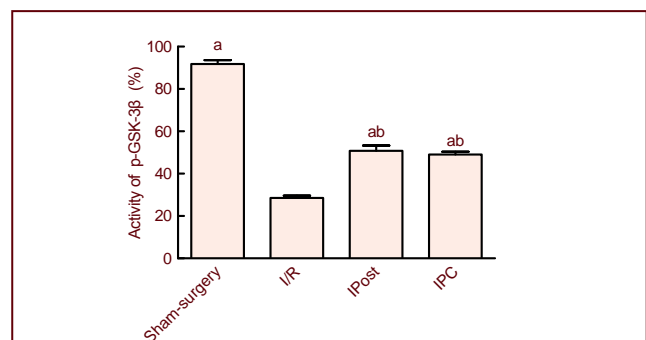### IPost increased the activity of phosphorylated-GSK-3β (p-GSK-3β) in the cerebral cortex (Figure 3)

Figure 3   IPost increased the activity of p-GSK-3β following global brain ischemia.

Each bar represents the mean ± SD of quadruplicate studies. The activity of p-GSK-3β was calculated using the following formula: (absorption × volume × dilution ratio)/ (total volume × absorbance index × 5).

[a]$P < 0.01$, *vs.* I/R group; [b]$P < 0.01$, *vs.* sham-surgery group. Different groups were compared by one-way analysis of variance and the Student-Newman-Keuls test.

I/R: Ischemia/reperfusion; IPost: ischemic postconditioning; IPC: ischemic preconditioning; p-GSK-3β: phosphorylated glycogen synthase kinase-3β.

As shown in Figure 3, the activity of p-GSK-3β in the cerebral cortex decreased markedly in the ischemia/ reperfusion group when compared to sham-surgery group rats, which was significantly restored by IPost and

ischemic preconditioning treatment ($P < 0.01$).

Correlation between TUNEL-positive cells, infract area and p-GSK-3β activity in the cerebral cortex

As shown in Figure 4, the activity of p-GSK-3β negatively correlated with the number of TUNEL-positive cells ($r = -0.80$) and infract area ($r = -0.77$). These data indicate that the increased phosphorylation of p-GSK-3β is attributable to the neuroprotection of IPost in the cerebral cortex.

Figure 4 Correlation between p-GSK-3β activity and the number of TUNEL-positive cells.

(A) Linear regression between TUNEL-positive cells and activity of p-GSK-3β ($r = -0.80$).

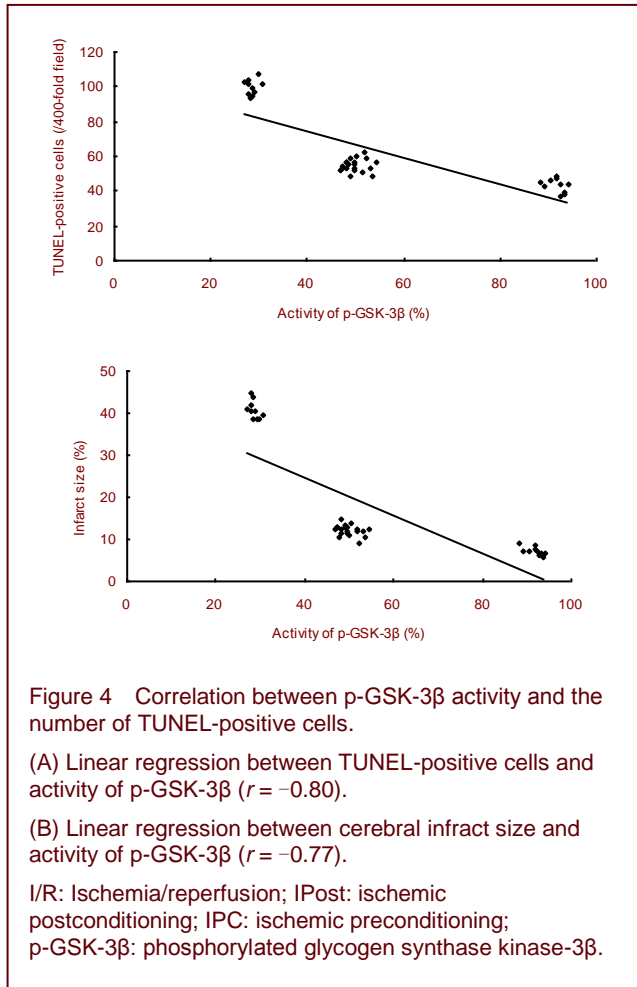(B) Linear regression between cerebral infract size and activity of p-GSK-3β ($r = -0.77$).

I/R: Ischemia/reperfusion; IPost: ischemic postconditioning; IPC: ischemic preconditioning; p-GSK-3β: phosphorylated glycogen synthase kinase-3β.
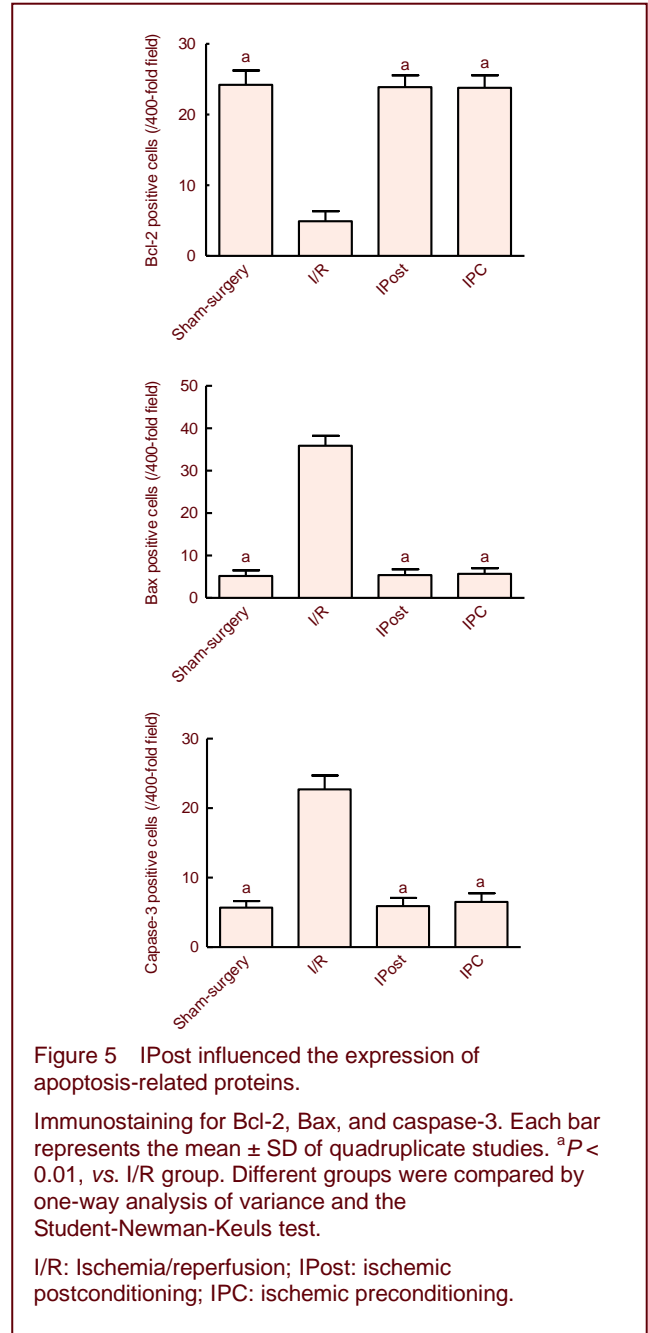
Influence of IPost on apoptosis-related proteins

Bcl-2, Bax and caspase 3 expression were observed by immunohistochemical staining in the ischemic cortex (supplementary Figure 2 online). As shown in Figure 5, Bcl-2 protein expression was significantly reduced in the ischemia/reperfusion group compared with the sham-surgery group rats ($P < 0.01$). Bcl-2 protein expression was significantly restored by IPost and ischemic preconditioning treatment, whereas Bax and caspase 3 expression increased significantly in the ischemia/reperfusion group when compared with the sham-surgery group, both of which were attenuated by IPost and ischemic preconditioning treatment ($P < 0.01$).

DISCUSSION

The present study showed that similar to ischemic preconditioning[10-11], cerebral IPost inhibited GSK-3β by enhancing p-GSK-3β activity, resulting in decreased infarct areas and cell apoptosis in the brain following global cerebral ischemia/reperfusion injury. The findings addressed the hypothesis that IPost provides neuronal protection by activating the GSK-3β-mediated antiapoptotic effect in the rat model of global ischemia.

Figure 5 IPost influenced the expression of apoptosis-related proteins.

Immunostaining for Bcl-2, Bax, and caspase-3. Each bar represents the mean ± SD of quadruplicate studies. [a]$P < 0.01$, *vs.* I/R group. Different groups were compared by one-way analysis of variance and the Student-Newman-Keuls test.

I/R: Ischemia/reperfusion; IPost: ischemic postconditioning; IPC: ischemic preconditioning.

Evidence suggests that both ischemic preconditioning and IPost, whether achieved by ischemia or by pharmacological means, appear to activate similar protective mechanisms in the heart and brain[12-13]. Apoptosis is a

crucial event in various physiologic processes, such as embryogenesis, organ development, and cell proliferation, as well as in pathologic processes, which contribute significantly to cell injury after ischemia/reperfusion injury[14-15]. A recent study has demonstrated that postconditioning exerts an anti-apoptotic effect both *in vivo* and *in vitro*[16]. Similarly, the present study showed that postconditioning significantly inhibited cell apoptosis in the cortex following ischemia/reperfusion cerebral injury. To further clarify the mechanisms of postconditioning protection, we investigated the expression of a few key cell death molecules. Results showed that postconditioning increased the level of the antiapoptotic Bcl-2 protein, and inhibited the protein expression of the proapoptotic molecules Bax and caspase-3. Bcl-2 is a potential mechanism for apoptotic resistance[17], which attenuates cellular injury and inhibits Bax translocation[18-19]. Active caspase-3 leads to DNA fragmentation[20]. Therefore, we speculate that IPost blocks ischemia/reperfusion-induced neuronal apoptosis in the cortex following ischemia/reperfusion cerebral injury through inhibiting the activation of caspase-3 and Bax and enhancing the activation of Bcl-2.

A previous study showed that the reperfusion injury salvage kinase pathway is the most important pathway in IPost. Within this pathway, PI3K/AKT plays a key role in blocking the apoptotic signaling pathway and GSK-3β has been shown to be a crucial mediator in the ischemic myocardium[21]. GSK-3β is highly conserved from yeasts to humans and is abundantly expressed in neural tissue[22]. When cells are injured, GSK-3β is phosphorylated or dephosphorylated (Ser9) to regulate the activities and functions of elements downstream in the pathway to maintain homeostasis[23-24]. Inhibition of GSK-3β subsequent to its phosphorylation reduces infarct volume and improves neurobehavioral functions[25]. Results from the present study showed that IPost enhanced p-GSK-3β activity, which correlated to reduced TUNEL-positive cells and infarct area after global cerebral ischemia. This suggests that GSK-3β expression increases following IPost and ischemic preconditioning, which results in neuroprotection. Moreover, GSK-3β may be a potential therapeutic target in preventing cerebral ischemia/ reperfusion injury.

In summary, in an *in vivo* model of cerebral ischemia/reperfusion, IPost protects against global cerebral ischemia/reperfusion injury by inhibiting apoptosis, and enhancing p-GSK-3β expression, which may represent a mechanism by which IPost confers neuroprotection.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting
The experiment was performed at the Laboratory of the Department of Anesthesiology, Renmin Hospital of Wuhan University, China from May to December, 2009.

Materials
Fifty-six adult male specific pathogen-free Wistar rats, weighing 200–230 g, were provided by the Centre for Disease Control and Prevention of Hubei Province, China (license No. SCXK (E) 2008-0005). All rats were housed under constant temperature 20 ± 2°C with 12-hour light/dark cycles. They were allowed free access to water and food.

Methods
Establishment of global brain ischemia model using the four-vessel occlusion method
The four-vessel occlusion method was applied as previously described[9]. In brief, rats were anesthetized with 10% (w/v) chloral hydrate (400 mg/kg, intraperitoneally). Vertebral arteries were cauterized with electricity and bilateral common carotid arteries were exposed and occluded by clamps. The clamps were removed to restore blood flow after 10 minutes of occlusion of the common carotid arteries, followed by 48 hours of reperfusion. Sham-surgery controls were operated with the same procedures without artery occlusion.

Ischemic preconditioning and IPost
IPost included three cycles of 30 seconds of reperfusion and 10 seconds of occlusion during the initial reperfusion; ischemic preconditioning involved three cycles of 10 seconds of occlusion and 30 seconds of reperfusion 10 minutes before global cerebral ischemia.

TUNEL staining for cell apoptosis in the rat cerebral cortex
TUNEL was used to identify cell apoptosis in the cerebral cortex[26]. Rats were sacrificed, and paraffin embedded sections were dewaxed and rehydrated, then incubated in 20 μL/mL proteinase K for 15 minutes. TUNEL was accomplished using an *in situ* cell death detection kit (Roche Inc, Frankfort, Germany). After immersion in equilibration buffer for 10 minutes, sections were incubated with TdT and dUTP-digoxigenin in a humidified chamber and then incubated in the stop/wash buffer. Sections were washed before incubation in anti-digoxigenin-peroxidase solution (1/500 in PBS), and colored with diaminobenzidine-H_2O_2 solution. TUNEL-positive cells had a brown color in the nucleus of dead cells. The number of TUNEL-positive cells in the cerebral cortex was quantified under the BX51 micro-

scope (× 400) using six randomly selected fields of view from five sections (Olympus, Shibuya, Japan).

2,3,5-triphenyltetrazolium chloride staining for brain infarct area

Rats were sacrificed at 48 hours after reperfusion and the brains were rapidly removed. Brains were cut into 2-mm-thick coronal sections using a cutting block[27-28] and stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) for 30 minutes at 37°C followed by overnight immersion in 10% (v/v) formalin. The infarcted brain remained unstained (white), whereas normal tissue stained red. The infarct areas were demarcated and analyzed using image analyzing software (Image-Pro Plus 6.0., Media Cybernetics, Inc., Silver Spring, MD, USA).

Detection of p-GSK-3β activity in the cerebral cortex

Forty-eight hours after reperfusion, fresh rat brain tissue was processed using the GSK-3β kinase kit (Genmed, Arlington, MA, USA). The degenerated brain tissue was centrifuged at $10\ 000 \times g$ at 4°C (Eppendorf Inc, New York, NY, USA), and the activity of p-GSK-3β was detected using a spectrophotometer (PE Inc, Waltham, MA, USA) at a wavelength of 340 nm.

Immunohistochemical staining for the expression of Bcl-2, Bax, and caspase-3 in the cerebral cortex

Immunohistochemistry was performed by staining for Bcl-2, Bax and caspase-3 in the cerebral cortex. Paraffin-embedded sections (10 rats from each group, six slices from each rat) were dewaxed and rehydrated. After immersion in equilibration buffer three times, sections were incubated with 3% (v/v) H_2O_2 and 10% (v/v) methanol in PBS[29] (pH 7.4) at room temperature in a humidified chamber for 10 minutes to block endogenous peroxidase activity. Sections were then incubated in stop/wash buffer and subjected to antigen retrieval by boiling sections in 10 mM sodium citrate buffer (pH 6.0) in a microwave for 20 minutes. After cooling sections to room temperature, slides were washed with PBS (pH 6.5). After being blocked in normal goat serum (dilution 1:10; Boster, Wuhan, China) for 10 minutes at room temperature, sections were incubated overnight at 4°C with rabbit anti-Bcl-2, Bax, or caspase-3 polyclonal antibodies (dilution 1:100; Santa Cruz Biotech Inc., Santa Cruz, CA, USA). After being washed with PBS, sections were incubated for 10 minutes at room temperature with biotinylated goat anti-rabbit IgG (dilution 1:10; Maixin Biotech Inc, Foochow, China) and washed with PBS, followed by incubation in a streptavidin-peroxidase complex (Maixin Biotech Inc.) for 10 minutes at room temperature. Staining was visualized using diaminobenzidine (Maixin Biotech Inc.) as a substrate. Negative control sections were not treated with primary antibodies. The number of positive cells was quantified under a BX51 microscope (× 400) using six random fields of view (Olympus).

Statistical analysis

All data were analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA) and figures were generated using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). All data are expressed as mean ± SD. The means of different groups were compared by one-way analysis of variance and the Student-Newman-Keuls test. Significant differences were identified by a value of $P < 0.05$.

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(Edited by Chen X, Luo XG/Su LL/Song LP)