Heme Oxygenase-1 Mediated Memorial and Revivable Protective Effect of Ischemic Preconditioning on Brain Injury

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SUMMARY

Aims: Ischemic preconditioning (IPC) has short-term benefits for stroke patients. However, if IPC protective effect is memorial and the role of the intracellular protective protein heme oxygenase-1 (HO-1) is not known. **Methods:** Ischemic preconditioning and the corresponding sham control were achieved by blocking the blood flow of the left internal carotid artery for 20 min and 2 second, respectively, in rats. Both IPC and sham-operated animals were divided into three groups and treated with PBS, the HO-1 inducer hemin, and the HO-1 inhibitor Znpp. Three weeks after IPC, brain ischemia–reperfusion injury was achieved by left middle cerebral artery obstruction for 45 min followed by 24-h reperfusion. **Results:** 2,3,5-triphenyltetrazolium chloride staining and neurological dysfunction scoring showed IPC significantly reduced brain infarct area and improved neurological function occurred 3 weeks after IPC. Hemin treatment promoted whereas ZnPP blocked the benefits of IPC. Immunohistochemical analysis and Western blotting showed that the expression of HO-1 was higher in the border zone than in the necrotic core zone. The memorial IPC protection is independent of adenosine receptor A1R and A2aR expressions. **Conclusions:** We found for the first time that the protective effect of IPC can be remembered to protect brain injury occurred after acute response disappear. The results indicate that interventional treatment can achieve protective effect for future cerebral injury not only through interventional treatment itself but also through the memorial and revivable IPC, eliminating the concern that temporary ischemia caused by interventional treatment may leave harmful effect in the brain.

Introduction

Stroke is the second most common cause of death worldwide and is a major cause of disability. Each year, approximately 20 million people experience a stroke [1]. Approximately 23.3% of stroke cases are those of recurrent stroke [2]. However, the impact of the first stroke attack on recurrent stroke is not known.

The current treatment of choice for stroke is thrombolysis. Because most patients with stroke do not receive professional treatment within 2 h of symptom onset [2], which is the time window for effective thrombolytic treatment, the use of interventional treatments for stroke has increased considerably. The interventional approaches used for the treatment and prevention of stroke include intra-arterial administration/use of therapeutic agents, gene vectors, cells, and stents and the use of devices to remove arterial clots. All these procedures require transient ischemia. The impact of therapeutic ischemia on injury related to stroke occurred in the future is also not known.

Preconditioning stimuli are diverse in nature. They can activate various pathways, altered gene translations and synthesis of pro-

teins. Ischemic preconditioning (IPC) is a common phenomenon wherein an ischemic event confers tolerance to subsequent ischemia [3]. Clinically, transient ischemic attacks induce tolerance by increasing the threshold of brain tissue vulnerability [4], which is critical for neuroprotection. Studies on IPC have shown that tolerance provides early (protection obtained minutes to hours after ischemia) or late (protection obtained hours to days after ischemia) protection in the brain [5].

The neuroprotective effects of IPC in the case of brain ischemia are at least partially mediated via heme oxygenase-1 (HO-1) [6]. HO-1 is the only rate-limiting inducible enzyme that can be induced to catalyze heme into antioxidant metabolic products, including biliverdin, carbon monoxide (CO), and iron and is popular for its cytoprotective functions such as its antioxidant, anti-inflammatory, anti-immune, antiapoptotic activities [7,8]. Hemin is specific HO-1 inducer [9] and protoporphyrin IX zinc (ZnPP) is selective HO-1 activity inhibitor [10].

Adenosine receptors are G-protein-coupled receptors, which are important protective molecules in IPC [11–13]. The subtypes of adenosine receptors are distributed diversely and in a complex

manner. Recently, research on the changes and effects of the adenosine receptors in brain ischemia has been mainly focused on two subtypes of the receptors: adenosine receptor A1 (A1R) and A2a (A2aR) [14]. Adenosine can activate adenosine receptor A1 on the postsynaptic membrane while acting as a neurotransmitter in nerve cells, and studies have shown that the short-term effect of brain IPC is mainly mediated by A1R [15]. The effect of A2aR on brain IPC is also reported [16]. However, if the regulation in the expression of adenosine receptors can be remembered remains unknown.

This study aimed to demonstrate if the protective effect of IPC can be remembered to protect brain ischemia injury occurred after acute response disappear. As both HO-1 and adenosine receptors are required for early IPC protective effect, the relationship between adenosine receptors and HO-1 in memorial IPC neuroprotection was also observed.

Methods

Animals

Twelve-week-old adult male Wistar rats weighing 200–250 g were used in this study. The animals were housed at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a 12 h:12 h light/dark cycle. Water and food were available *ad libitum*. All the protocols were approved by the institutional animal care and use committee of Shanghai Medical College, Fudan University, and performed in accordance with the NIH guidelines for the care and use of laboratory animals.

Animal Model of Left Brain IPC

The animals were anesthetized with 10% hydrated chlorine aldehyde via intraperitoneal injection. The blood flow of the left internal carotid artery was stopped for 20 min in the experimental group and for 2 second in the sham group. Middle cerebral artery ischemia injury (MCAI) was performed 3 weeks after initial ischemia (IPC). The procedures used for MCAI were as follows: under the operating microscope, a small incision was made in the left external carotid artery. A nylon filament (diameter, 0.36 mm) was inserted into the left internal carotid artery through the opening of the left external carotid artery and advanced further to occlude the left middle cerebral artery (LMCA) for 45 min. The nylon filament was then removed, and the left external carotid artery was tied. Twenty-four hours after the blood flow of the left internal carotid artery was restored, the animals were sacrificed for examination.

Evaluation of Infarct Size and Neurological Deficit Score

Twenty-four hours after reperfusion, the rats were deeply anesthetized with isoflurane (5% in O_2), and the brain was perfused with heparinized saline (10 U/mL) *in situ* for approximately 90 second. Each brain was sliced into 2-mm-thick sections using a brain matrix (BSR 001.1; Zivic-Miller, Pittsburgh, PA, USA). The third section of each brain was stained with 0.5% 2,3,5-triphenyl-tetrazolium chloride (TTC) for 20 min at 37°C , followed by fixation in 4% formaldehyde at 4°C for 24 h. The second and fourth

of the 2-mm-thick coronal brain sections, located at 2–4 mm and 6–8 mm, respectively, from the frontal region were stored at -80°C for future study. The relative infarct area (% of total left hemisphere) of the third section was measured using Image-Pro plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

Neurological Function Analysis

The rats were assessed for neurological dysfunction using a modified Bederson score [17] in a double-blinded manner with the following definitions:

- Score 0: no detectable neurological deficits
- Score 1: body torsion present
- Score 2: body torsion with right-sided weakness
- Score 3: body torsion and right-sided weakness with circling behavior
- Score 4: unresponsiveness, that is, a low level of consciousness and absence of active movements
- Score 5: death before scheduled termination.

Drugs and Experimental Groups

The HO-1 inducer hemin and the HO-1 inhibitor Znpp (protoporphyrin IX zinc) were purchased from Sigma (St. Louis, MO, USA). The rats were randomly divided into two groups: experimental group, that is, ischemia 20 min; and sham control group, that is, ischemia 2 seconds. Both the experimental and control groups were divided into three subgroups that received PBS, hemin or Znpp. PBS, hemin (10 $\mu\text{mol/kg}$) or Znpp (10 $\mu\text{mol/kg}$) were injected subcutaneously once every 3 days, starting 1 days before IPC. Three weeks after IPC, all the animals were subjected to MCAI.

Immunohistochemistry

After evaluation of the infarct size and the neurological dysfunction score, 5- μm -thick coronal sections of the previously fixed third brain matrix section were used for immunohistochemical studies. The sections were incubated in 3% H_2O_2 for 10 min, followed by 3 washes in 0.01 M PBS [18]. The sections were then blocked with 10% donkey serum (Jackson, USA) following incubation with the rabbit anti-HO-1 (1:100, Abcam, Cambridge, MA, USA), antiadenosine receptor A1 (1:100, Abcam), or antiadenosine receptor A2a (1:10, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody. The sections were subsequently incubated overnight with the primary antibodies at 4°C ; subsequently, they were washed and incubated with HRP-labeled donkey anti-rabbit IgG secondary antibodies for 30 min at 37°C . Diaminobenzidine (DAB) was used for visualization of the sections. The slides were counterstained slightly with hematoxylin before dehydration and mounting.

Western Blotting

Frozen samples were used for Western blot-based quantification of the HO-1, adenosine receptor A1 and A2a proteins. Tissues from ischemic brains were homogenized in a buffer containing

4% SDS, 0.1 M Tris-Cl, 20% glycerin, and 0.01 mM PMSF, pH 7.4. The homogenates were centrifuged at 10,000 g at 4°C for 20 min. The supernatant was used as the cytosolic fraction. The protein concentration was quantified using the BCA kit (Beyotime, Shanghai, China). Thirty micrograms of cytosolic protein was run on a 0.1% SDS–10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. To block nonspecific protein binding, the membranes were incubated with 5% dehydrated skim milk and in PBS containing 0.05% Tween20. The primary antibodies were incubated overnight with the membranes at 4°C; then, the secondary antibodies were incubated with the membranes for 1 h at room temperature. The enhanced chemiluminescence detection system kit (General Bioscience Corporation, Brisbane, CA, USA) was used for antibody detection. The membranes were exposed to X-ray film to record chemiluminescence. For quantitative analysis, the bands were scanned and analyzed with a laser densitometer (Tanon, UVP, Upland, CA, USA).

Statistical Analysis

Statistical analysis was performed using ANOVA followed by the *t*-test, except in the case of neurobiological dysfunction score,

which were analyzed by the nonparametric Kruskal–Wallis test. All the data have been provided in terms of mean $\pm$ SEM values. *P* values less than 0.05 were considered statistically significant.

Results

Prolonged Memory-based Protection Because of IPC in Brain Injury and the Role of HO-1

The TTC staining results showed that the infarct zone was concentrated in the striatum of the left hemisphere of the brain, with a portion extending to the cortex. The IPC 20 min + PBS ($n = 9$) treatment decreased the infarct size (Figure 1) and the neurological dysfunction score (Figure 2) caused by MCAI induced 3 weeks after IPC more significantly than the IPC 2S + PBS ($n = 9$, $P < 0.05$) and IPC 20 min + Znpp treatments ($n = 9$, $P < 0.05$) did. The IPC 20 min + hemin pretreatment ($n = 9$) decreased the MCAI-induced infarct size and neurological dysfunction score more significantly than the IPC 20 min + PBS or Znpp ($P < 0.05$) did. The preventive effects of IPC 20 min or hemin alone were similar. ZnPP blocked the IPC 20 min-mediated protection with respect to MCAI-induced infarct size (Figure 1) and neurological dysfunction score (Figure 2).

Figure 1 Memorial ischemic preconditioning decreased infarct area caused by brain injury. (A) Triphenyltetrazolium chloride (TTC) staining of the third brain slice of all animals. (B) Measurement of the infarct size of third brain slice. Data are reported as mean $\pm$ SEM. *represents $P < 0.05$ two groups with different treatment but with same ischemic preconditioning (IPC) time; $n = 9$.

Figure 2 Memorial ischemic preconditioning decreased neurological dysfunction caused by brain injury. Data are reported as mean $\pm$ SEM. #represents $P < 0.05$ between ischemic preconditioning (IPC) 20 min plus and IPC 2 seconds plus PBS. *represents $P < 0.05$ two groups with different treatment but with same IPC time; $n = 9$.

HO-1 Expression

All the brain sections showed positive results for the HO-1 protein immunohistochemical staining. The HO-1 protein was mainly distributed in the infarct marginal zone in the striatum and cortex instead of the infarct zone (Figure S1). HO-1 expression increased in all groups after MCAI, except the IPC 2S + Znpp group (Figure S1).

Western blot analysis showed that both IPC 20 min and HO-1 inducer hemin increased HO-1 expression on both infarct and infarct marginal regions, respectively (Figure 3). Furthermore, inductions of IPC 20 min and hemin on HO-1 expression were synergic in infarct marginal region (Figure 3A,B). HO-1 activity

competitive inhibitor ZnPP did not affect HO-1 expression (Figure 3).

Adenosine Receptor A1 Expression

Intense A1R immunohistochemical staining was found in the cortex of the adult rat brain (Figure S2). In the infarct marginal zone, Western blotting analysis showed that HO-1 was higher in IPC 20 min groups than in IPC 2S groups. However, no difference was observed between PBS, hemin, and ZnPP treatments (Figure 4A, B). In the infarct zone, no difference in A1R expression was found among the groups (Figure 4C,D). However, we found an additional A1R band in samples from infarct region (Figure 4C). This extra band was not observed in samples from infarct marginal region (Figure 4A).

Adenosine Receptor A2aR Expression

A2aR staining was intense in the striatum of the adult rat brain (Figure S3). Western blotting analysis showed that A2aR protein expression was greater in the infarct marginal zone than in the infarct zone. The A2a expression of the different groups did not significantly differ in the infarct marginal zone (Figure 5).

Discussion

Recurrent stroke is a common phenomenon in stroke patients. IPC has been shown to provide protective effects against brain injury in hours to days after it is initiated. Most of the protective mechanisms of IPC are related to an acute protective response against acute injury or inflammation. However, recurrent stroke in most patients occurs after the inflammatory response disappears. Whether IPC induced by initial stroke is still protective against recurrent stroke that occurs after acute inflammatory response disappears is unknown.

Figure 3 Heme oxygenase-1 in infarct and marginal zones. HO-1 was detected by Western blotting. (A & C) Images of Western blotting for the samples from infarct marginal and infarct regions, respectively. (B & D) Densitometry of HO-1 in infarct marginal and infarct regions, respectively. Data are reported as mean $\pm$ SEM. #represents $P < 0.05$ between two groups with same treatment but different ischemic preconditioning (IPC) time (20 min and 2 seconds). *represents $P < 0.05$ two groups with different treatment but with same IPC time; $n = 9$.

Figure 4 Adenosine receptor adenosine receptor A1 (A1R) in infarct and marginal zones. Adenosine receptor A1R was detected by Western blotting. (A & C) Images of Western blotting for the samples from infarct marginal and infarct regions, respectively. (B & D) Densitometry of A1R in infarct marginal and infarct regions, respectively. Data are reported as mean $\pm$ SEM. #represents $P < 0.05$ between two groups with same treatment but different ischemic preconditioning (IPC) time (20 min and 2 seconds); $n = 9$.

Figure 5 Adenosine receptor A2aR in infarct and marginal zones. Adenosine receptor A2aR was detected by Western blotting. (A & C) Images of Western blotting for the samples from infarct marginal and infarct regions, respectively. (B & D) Densitometry of A2aR in infarct marginal and infarct regions, respectively. Data are reported as mean $\pm$ SEM; $n = 9$.

Because the current treatments for stroke are unsatisfactory, many new therapeutic interventions, including intra-arterial administration/use of drugs, gene vectors, cells, or stents and the use of devices to remove arterial clots, are being developed. It is critical to determine whether the transient ischemia required for these procedures is protective or harmful with respect to recurrent stroke that occurs long-term after. Our study demonstrated that IPC-induced neuroprotection in the brain was memorial and can be revived by future MCAI. The memory response has been reported earlier in other cases. For example, memory B lymphocytes are responsible for the memory of the humoral immune response. The memory response can also be seen in the case of recurrent drug or tobacco addiction. Because the memorial protection can be revived by future injury occurs long-term after induction, it is different from prolonged IPC protection, in which injury occurs within hours to days after IPC but lasts for a long time.

There are various possible explanations for the protective effect of IPC against brain ischemia. IPC triggers multiple mechanisms including HO-1 induction that eventually dictate cellular responses to more severe injury. HO-1 is an important intracellular protective enzyme regulated by many intracellular molecules

[19,20]. It can be induced by many natural and pharmacological inducers [19–21]. For example, the transcriptional factor hypoxia-inducible factor 1 α (HIF-1 α) activates HO-1 to further regulate early and delayed IPC protective mechanisms. HO-1 upregulation induced by HIF-1 α after I/R injury improves cell survival and activates angiogenesis pathways, including those involved in collateral vessel development, and also occurs in a tissue-specific manner. HO-1 metabolizes the pro-oxidant heme, present in toxic levels, to produce CO, antioxidants, the free radical scavengers biliverdin and bilirubin, and iron. These products of HO-1 can attenuate inflammation and apoptotic responses after ischemia [10,22,23]. Because HO-1 is important in IPC protection for stroke that occurs immediately (in hours to days) after IPC, we wondered if the IPC-induced HO-1 expression shows a memory-based response, that is, quicker and stronger responses to subsequent stimulation. To determine the role of HO-1 in the memory of IPC neuroprotection, the HO-1 inhibitor ZnPP was administered before MCAI, and it was found that this inhibitor can block IPC neuroprotection. In the meantime, HO-1 inducer mimicked the memory response of IPC.

Adenosine plays a neuroprotective role in the ischemic brain through A1R and A2aR [14,24]. A1R has been shown to be an

endogenous protein mediating protection when induced by different preconditioning stimuli applied to various organs. Administration of an A1R agonist attenuated neural damage both *in vivo* and *in vitro* [14,24]. Further, an A1R antagonist completely blocked IPC protection, whereas an A2aR antagonist blocked it partially [25]. To determine if A1R and A2aR are involved in HO-1-mediated memory-based IPC protection, we measured their protein expression using Western blot analysis and immunohistochemical assays. We found that neither HO-1 induction nor HO-1 inhibition changed A1R and A2aR expression. IPC did not change A2aR expression. In the meantime, although IPC increased A1R expression, A1R level was not correlated with memorial IPC protection. We also found that there is only one A1R band in the western gel image of samples collected from infarct marginal region. However, there are two A1R bands in the western gel of samples collected from infarct region. The mechanism underlining the difference is not known. Because the phenomenon is not seen in HO-1 and A2aR, mechanisms other than protein analysis may involve. Dephosphorylation and demethylation may be two of the possible mechanisms. Similar to A1R expression, the number of A1R band was not correlated with memorial IPC protection. Thus, memorial IPC protection is independent of A1R and A2aR expression. These results indicated that only some of the mechanisms underlying

short-term IPC protection (for example, HO-1 activity) are involved in memorial IPC protection.

In summary, we found, for the first time, that the protective effect of IPC can be remembered to protect against brain ischemia injury even after disappearance of the acute response. HO-1 is required for prolonged memorial protection. Our result indicates that temporary ischemia caused by interventional treatment for stroke may have the benefit of not only short-term but also long-term protection against brain injury.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Immunohistochemical analysis of HO-1.

Figure S2. Immunohistochemical analysis of adenosine receptor A1R.

Figure S3. Immunohistochemical analysis of adenosine receptor A2aR.