Role of phosphoinositide 3-kinase in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked behavioral deficits in mice

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Abstract:
The present study has been designed to pharmacologically investigate the role of phosphoinositide 3-kinase in ischemic postconditioning-induced reversal of global cerebral ischemia and reperfusion-induced behavioral dysfunction in mice. Bilateral carotid artery occlusion for 10 min followed by reperfusion for 24 h was employed in the present study to produce ischemia and reperfusion-induced cerebral injury in mice. Short-term memory was evaluated using the elevated plus maze test. The inclined beam walking test was employed to assess motor incoordination. Bilateral carotid artery occlusion followed by reperfusion produced impaired short-term memory, motor co-ordination and lateral push response. Three episodes of carotid artery occlusion for a period of 10 s and reperfusion of 10 s (ischemic postconditioning) significantly prevented ischemia-reperfusion-induced behavioral deficit measured in terms of loss of short-term memory, motor coordination and lateral push response. Wortmannin (2 mg/kg, iv), a phosphoinositide 3-kinase inhibitor given 10 min before ischemia attenuated the beneficial effects of ischemic postconditioning. It may be concluded that beneficial effects of ischemic postconditioning on global cerebral ischemia and reperfusion-induced behavioral deficits may involve activation of phosphoinositide 3-kinase-linked pathway.

Key words:
ischemic postconditioning, cerebral ischemia, phosphoinositide 3-kinase, wortmannin

Introduction

Ischemic stroke is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain [3]. Although mortality from ischemic stroke has declined over the last decade, it remains the third leading cause of death, as only limited therapeutic strategies exist [23]. Ischemic preconditioning is a potent protective strategy introduced by Murray and coworkers [18] for the ischemic myocardium which was later applied by Kitagawa et al. to the ischemic neuronal injury as well [17]. Ischemic post-conditioning refers to protective effect of brief episodes of ischemia-reperfusion immediately after a more severe form of ischemic insult to an organ [27]. Protective effect of ischemic postconditioning was produced in the ischemic myocardium as well as in the brain by short cycles of ischemia and reperfusion immediately after a prolonged ischemic injury in multiple animal models [15, 16, 26, 27]. Ischemic postconditioning has recently been shown to be effective in limited clinical studies on human subjects, as well [21]. The activation of phosphoinositide 3-kinase (PI-3-K)-linked transduction pathway has been implicated in the cardioprotective manifestations of ischemic post-
conditioning of the myocardium [19]. Wortmannin selectively and irreversibly binds to and inhibits PI-3-K [2]. Therefore, the present study has been designed to pharmacologically investigate the role of phosphoinositide 3-kinase in ischemic postconditioning-induced reversal of global cerebral ischemia and reperfusion-induced behavioral dysfunction in mice.

Materials and Methods

Animals

Male albino Swiss mice weighing 25 ± 2 g, maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and having free access to tap water were employed in the present study. They were housed in the departmental animal house and were exposed to 12-h light/dark cycle. All the animals used in the study were naive to elevated plus-maze test. The experiments were conducted in a semi-sound proof laboratory. The animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. – 107/1999/CPCSEA).

Drugs and chemicals

Wortmannin (Tocris, Missouri 63021, USA) and chloral hydrate (Riedel-de Haen, Germany) were dissolved in 10% dimethylsulfoxide in physiological saline and normal physiological saline, respectively. All other chemicals used in the present study were of analytical quality. All drug solutions were freshly prepared before use.

Induction of global cerebral ischemia

Global cerebral ischemia was induced by occluding the carotid arteries [12]. After 10 min of global cerebral ischemia, reperfusion was allowed for 24 h. The incision was sutured back in layers. The sutured area was cleaned with 70% ethanol and was sprayed with antiseptic dusting powder. The animals were shifted individually to their home cage and were allowed to recover.

For the ischemic postconditioning episode, the carotid arteries were re-occluded for a period of 10 s followed by 10 s of reperfusion time. Three such cycles of ischemia and reperfusion were allowed immediately after the bilateral carotid artery occlusion performed for 10 min.

Evaluation of short-term memory using elevated plus maze test

Plus maze consisted of two open (16 × 5 cm) and two closed (16 × 5 × 12 cm) arms, connected by a central platform (5 × 5 cm). The apparatus was elevated to a height of 25 cm above the floor. A fine line was drawn in the middle of the floor of each closed arm. All the animals were given a single trial/day on plus maze. Each mouse was individually placed at the end of an open arm facing away from central platform of the maze. The time taken by the mouse to enter from open arm with all the four legs into the closed arm was taken as transfer latency time (TLT). In case the animal did not enter the closed arm within 90 s, it was gently pushed into the closed arm and TLT of 90 s was assigned to it. The animal was allowed to explore the maze for an additional 10 s after the measurement of TLT [11, 13]. The animal was examined in the elevated plus maze test for three consecutive days [25]. TLT recorded on the third day served as an index of acquisition whereas TLT measured on day four served as an index of memory. After recording day 3 TLT, the animal was subjected to global cerebral ischemia for 10 min followed by reperfusion for 24 h and was again examined in the elevated plus maze test. Utmost care was taken not to change the relative location of plus maze with respect to any object serving as visual clue in laboratory.

Inclined beam-walking test

Inclined beam-walking test was employed to evaluate fore and hind limb motor coordination [10, 11]. Each animal was individually placed on a metallic bar 55 cm
long and 1.5 cm wide, inclined at an angle of 60° from ground. The motor performance of mouse was scored on a scale ranging from 0 to 4. A grade of 0 was assigned to animal that could readily traverse the beam, grade 1 was given to animal demonstrating mild impairment, grade 2 was assigned to animal demonstrating moderate impairment, grade 3 was given to animal demonstrating severe impairment and grade 4 was assigned to animal completely unable to walk on the beam. Inclined beam-walking test was performed before global cerebral ischemia and 12 and 24 h after global cerebral ischemia and reperfusion.

**Lateral push test**

A mouse was placed on a rough surface for firm grip and was evaluated for resistance to lateral push from either side of shoulder [5, 11]. The test was performed before global cerebral ischemia and 12 and 24 h after global cerebral ischemia and reperfusion. Mice with increased or decreased resistance to lateral push after global ischemia were assigned + or – score respectively.

**Experimental protocol**

In total, five groups were employed and each group comprised 10 animals.

**Sham group (Group I)**

Each mouse was subjected to surgical procedure and carotid arteries were isolated and thread was passed below it but the arteries were not occluded. After 10 min, thread was removed and the animal was sutured back and allowed to recover for 24 h.

**Control group (Group II)**

Each mouse was subjected to surgical procedure and to 10 min global cerebral ischemia followed by reperfusion for 24 h.

**Ischemic postconditioning group (Group III)**

Each mouse was subjected to 10 min global cerebral ischemia followed by three episodes of 10 s of ischemia and reperfusion after which a 24-h reperfusion period was permitted.

**Wortmannin alone group (Group IV)**

Each mouse was administered wortmannin (2 mg/kg, iv) 10 min prior to carotid artery isolation. The rest of the procedure was the same as described for group II.

**Wortmannin-treated postconditioning group (Group V)**

Each mouse was administered wortmannin (2 mg/kg, iv) 10 min prior to carotid artery occlusion. The rest of the procedure was the same as described for group III (Fig. 1).

**Statistical analysis**

Statistical analysis for infarct size and TLT was done using one-way ANOVA followed by Dunett’s test and Tukey’s multiple range test as *post-hoc* analysis. Statistical significance for lateral push and beam walking were calculated using Chi square and Wilcoxon rank sum test, respectively. A value of p < 0.05 was considered to be statistically significant.

**Results**

**Effect on transfer latency time**

Sham group animals showed a significant decrease in day 4 TLT when compared to their day 3 TLT. Global cerebral ischemia followed by reperfusion significantly prevented the decrease in day 4 TLT reflecting impairment of memory. Ischemic postconditioning significantly attenuated ischemia-reperfusion-induced increase in day 4 TLT of control indicating reversal of ischemia-reperfusion induced impairment of memory. Wortmannin (2 mg/kg, iv) *per se* did not modulate ischemia-reperfusion-induced increase in day 4 TLT. However, it significantly attenuated ischemic postconditioning-induced decrease in day 4 TLT (Fig. 2).

**Effect on motor incoordination**

Global cerebral ischemia followed by reperfusion produced significant motor incoordination in mice noted after 12 and 24 h of reperfusion when compared to sham group. Ischemic postconditioning markedly prevented ischemia-reperfusion induced motor incoordination. Wortmannin (2 mg /kg, iv) *per se* did not
affect ischemia-reperfusion-induced motor incoordination. However, it significantly attenuated ischemic postconditioning-induced decrease in motor incoordination (Fig. 3).

**Effect on lateral push response**

Global cerebral ischemia followed by reperfusion produced a significant decrease in percentage of mice demonstrating resistance to lateral push noted after 12 and 24 h of reperfusion, when compared to sham group. Ischemic postconditioning significantly prevented ischemia-reperfusion-induced decrease in percentage of mice demonstrating resistance to lateral push. Wortmannin (2 mg/kg, iv) *per se* did not modify ischemia-reperfusion-induced decrease in percentage of mice demonstrating resistance to lateral push. However, it significantly attenuated ischemic postconditioning induced increase in percentage of mice demonstrating resistance to lateral push (Fig. 4).

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**Fig. 1.** Schematic representation of the experimental protocol
Discussion

Global cerebral ischemia and reperfusion model employed in the present study has been reported to simulate the clinical condition of cerebral ischemia [1]. Global cerebral ischemia has been reported to impair short-term memory because hippocampal neurons are susceptible to the deleterious effects of ischemia and reperfusion [14] and the hippocampus is involved in regulation of short-term memory. Cerebral ischemia has been further documented to impair sensorimotor ability, as well [9]. Therefore, in the present investigation, we employed the elevated plus-maze test to assess short-term memory, and inclined beam walking test and resistance to lateral push test for evaluation of sensorimotor ability. In our study, global cerebral ischemia, reperfusion induced impairment of short-term memory, as well [9].

Fig. 2. Effect of ischemic postconditioning and wortmannin on ischemia and reperfusion-induced impairment of short-term memory in mice. Values are the mean ± SEM. Statistical analysis for TLT was done using one-way ANOVA followed by Dunnett’s test and Tukey’s multiple range test as post hoc analysis. a p < 0.05 vs day 3 TLT of sham; b p < 0.05 vs day 4 TLT of sham; c p < 0.05 vs day 4 TLT of control; d p < 0.05 vs day 4 TLT of ischemic postconditioning.

Fig. 3. Effect of ischemic postconditioning and wortmannin on ischemia and reperfusion-induced impairment of motor coordination noted after 12 and 24 h of reperfusion in mice. Values are the mean ± SEM. Wilcoxon rank sum test was used to test the statistical significance of difference between various groups. a p < 0.05 vs sham; b p < 0.05 vs control; c p < 0.05 vs ischemic postconditioning.

Fig. 4. Effect of ischemic postconditioning and wortmannin on ischemia and reperfusion-induced impairment of resistance to lateral push noted after 12 and 24 h of reperfusion in mice. Values are percentage of mice showing resistance to lateral push. Chi-square test was used to test the statistical significance of difference between various groups. a p < 0.05 vs sham; b p < 0.05 vs control; c p < 0.05 vs ischemic postconditioning.
term memory as well as of motor coordination. These findings are in line with an earlier report [11]. Further, cerebral ischemia-reperfusion-induced impairment of the behavioral manifestations are prevented by ischemic postconditioning. These findings are consistent with a recent report of postconditioning-evoked partial prevention of cerebral ischemic injury in rats [6, 8, 26], and extend them reporting functional neuroprotection. In the present investigation, wortmannin per se did not alter ischemia-reperfusion induced behavioral impairment; however, it abolished the protective effect of ischemic postconditioning. Some recent studies have indicated a potential role of PI-3-K in brain preconditioning and in cardioprotective effect of ischemic postconditioning, which may be mediated through activation of PI-3-K-eNOS-Akt pathway [7, 20, 22]. This contention is further supported by our study in which wortmannin, a selective inhibitor of PI-3-K [2], attenuated the protective effect of ischemic postconditioning. Therefore, it may be suggested that beneficial effects of ischemic postconditioning in global cerebral ischemia and reperfusion-induced behavioral deficits may be due to the activation of PI-3-K-linked transduction pathway. Akt is an initiator of downstream pathways that inhibit the apoptotic routes by inhibiting the release of cytochrome c due to blocking the channel formation in the mitochondrial membrane by Bax [4, 24].

On the basis of the above discussion, it may be concluded that ischemic postconditioning exerts beneficial effect on behavioral dysfunction possibly through a PI-3-K-linked mechanism. Nevertheless, further studies are required to elucidate the involvement of phosphoinositide 3-kinase in the neuroprotective manifestations of ischemic postconditioning.

Acknowledgments:
The authors are grateful to Dr. Ashok Kumar Tiwary, Head of Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India for supporting this study and providing technical facilities for the work.

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Received: January 4, 2007; in revised form: April 22, 2007.