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Pharmacologic evidence for role of endothelial nitric oxide synthase in neuroprotective mechanism of ischemic postconditioning in mice

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ABSTRACT

Background: The present study was conducted to pharmacologically investigate the isoform-specific role of nitric oxide pathway in neuroprotective mechanism of ischemic postconditioning (iPoCo).

Materials and methods: Bilateral carotid artery occlusion of 12 min followed by reperfusion for 24 h was used to produce ischemia- and reperfusion-induced cerebral injury in male Swiss mice. Memory was assessed using Morris water maze test. Degree of motor in-coordination was evaluated using inclined beam-walk test, rotarod test, and lateral push test. Cerebral infarct size was measured using triphenyltetrazolium chloride staining. Brain acetylcholinestrase activity, thiobarbituric acid–reactive species, nitrite/nitrate, and reduced glutathione levels were also estimated. Western blotting was performed to determine endothelial nitric oxide synthase (eNOS) expression.

Results: Bilateral carotid artery occlusion followed by reperfusion produced significant rise in cerebral infarct size, acetylcholinesterase activity, and thiobarbituric acid–reactive species levels along with fall in nitrite/nitrate, and glutathione and eNOS expression levels. A significant impairment of memory and motor coordination was also noted. iPoCo consisting of three episodes of 10-s carotid artery occlusion and reperfusion significantly attenuated infarct size, memory impairment, motor in-coordination, altered biochemicals, and protein expression levels. iPoCo-induced neuroprotective effects were significantly abolished by L-NAME (a nonselective nitric oxide synthase inhibitor) and L-NIO (a selective eNOS inhibitor). However, aminoguanidine (a selective inducible nitric oxide synthase inhibitor) and 7-nitroindazole (a selective neuronal nitric oxide synthase inhibitor) did not modulate beneficial effects of iPoCo.

Conclusions: It may be concluded that nitric oxide pathway probably plays a vital role with specific involvement of eNOS in neuroprotective mechanism of iPoCo.

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1. Introduction

Ischemic stroke is the acute severe manifestation of cerebrovascular disease. It is a syndrome characterized by rapid onset of neurologic injury due to interruption of blood flow to the brain [1,2]. Although mortality from ischemic stroke has declined over the last decade, it still remains the second leading cause of death because only limited therapeutic strategies exist [3]. Restoration of blood flow to ischemic tissue is the only way to reduce infarct size, but full reperfusion itself results in additional injury known as ischemia-reperfusion (I/R) injury [4]. An attempt to attenuate this injury led to the discovery of phenomenon like ischemic preconditioning (iPreCo) and ischemic postconditioning (iPoCo) [5]. iPreCo is a well-established phenomenon, being used since 1980s to attenuate I/R-induced injury [6,7]. However, inability of preconditioning phenomenon to predict the onset of ischemia in clinical settings [8] led to the discovery of a new concept of iPoCo, whereby brief repetitive cycles of ischemia with intermittent reperfusion immediately after prolonged ischemia elicit tissue protection [5,8,9]. Protective role of iPoCo has been documented in different organs like heart of several animal species [10,11], brain [9,12,13], liver [14], and kidney [15]. iPoCo is also observed to produce cardioprotective effects in clinical settings [16,17].

There is an impressive array of molecular mechanisms contributing to protective effect of iPoCo which include triggers like adenosine, opioid, erythropoietin, endothelial nitric oxide synthase (eNOS), reactive oxygen species, acetylcholine, tissue factors, proinflammatory cytokines, and bradykinin; mediators like RISK pathways including PI3K-Akt, MEK-ERK½, PKG, and PKC; end-effectors like mPTP and mKATP [18].

Nitric oxide (NO), also known as endothelium-derived relaxing factor, is a key biological messenger playing a prominent role in preserving the functions of endothelium. The human genome contains three different genes encoding NO synthases (neuronal nitric oxide synthase [nNOS], inducible nitric oxide synthase [iNOS], and eNOS). A protective effect of NO has also been documented during reperfusion of ischemic myocardium [19]. It has been observed that eNOS plays an important role in mediating cardioprotective effects of iPoCo [20] and both eNOS and iNOS are involved in renal iPoCo [15]. Furthermore, it is also reported that NO is also involved in cerebroprotective effect of delayed preconditioning. However, role of nitric oxide synthase (NOS) in postconditioning mediated neuroprotection still remains to be evaluated.

Therefore, the present study has been designed to pharmacologically investigate the specific role of NOS isoform in iPoCo-induced reversal of global cerebral ischemia- and reperfusion-induced injury in mice.

2. Experimental

2.1. Animals

Male Swiss mice weighing 20 ± 5 g, maintained on standard laboratory diet (Kisan Feeds Ltd, Mumbai, India) and having free access to tap water were used 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 Morris water maze (MWM) test. The experiments were conducted in a semisound proof laboratory. The experimental protocol was duly approved by institutional animal ethics committee. The care of the animals was 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).

2.2. Drugs and chemicals

L-NAME (Cayman Chemicals, Ann Arbor, MI), L-NIO (Cayman Chemicals, Ann Arbor, MI), aminoguanidine (Sigma-Aldrich Ltd, St Louis, MO), and chloral hydrate (Riedel-deHaen, Seelze Germany) were dissolved in distilled water, respectively. 7-Nitroindazole (7-NI) (Cayman Chemicals) was dissolved in 5% dimethyl formamide (DMF) solution. Anti-eNOS antibody (EMD chemicals, Darmstadt, Germany) and goat anti-mouse IgG peroxidase conjugate (EMD chemicals) were used for western blotting. All other chemicals used in the present study were of analytical quality. All drug solutions were freshly prepared before use. The drugs were administered 30 min before carotid ligation (ischemia) to the animals. This schedule of drug administration was adapted to ensure maximum drug absorption just before the induction of iPoCo.

2.3. Induction of global cerebral ischemia

Mice were anesthetized using chloral hydrate (400 mg/kg, intraperitoneally [i.p.]) as mortality is negligible at this dose and at the same time cognitive function is not at all affected [3,9,12,21,22]. Global cerebral ischemia in mice was induced surgically according to the methods of Himori *et al.* [23] and as described by Kaur *et al.* [12]. A midline ventral incision was made in the neck to expose right and left common carotid arteries, which were isolated from surrounding tissue and vagus nerve. A cotton thread was passed below each of the carotid arteries. Global cerebral ischemia was induced by occluding the carotid arteries. After 12 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.

2.4. Induction of iPoCo cycles

For the iPoCo episode, the carotid arteries were reoccluded 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 (BCAO) performed for 12 min [9].

2.5. Assessment of cerebral infarct size

At the end of 24 h of reperfusion after global cerebral ischemia, animals were killed by spinal dislocation, and the