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Original Contribution

Ischemic preconditioning protects hippocampal pyramidal neurons from transient ischemic injury via the attenuation of oxidative damage through upregulating heme oxygenase-1

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ABSTRACT

Ischemic preconditioning (IPC) provides neuroprotection against subsequent severe ischemic injury by activating specific mechanisms. In this study, we tested the hypothesis that IPC attenuates postischemic neuronal death via heme oxygenase-1 (HO-1). Animals used in this study were randomly assigned to 4 groups; sham-operated group, ischemia-operated group, IPC plus (+) sham-operated group and IPC+ischemia-operated group. IPC was induced by subjecting gerbils to 2 min of ischemia followed by 1 day of recovery. A significant loss of neurons was observed in pyramidal neurons of the hippocampal CA1 region (CA1) in the ischemia-operated groups at 5 days postischemia. In the IPC+ischemia-operated groups, CA1 pyramidal neurons were well protected. The level of HO-1 protein and its activity increased significantly in the CA1 of the IPC+sham-operated group, and the level and activity was maintained in all the time after ischemia–reperfusion compared with the ischemia-operated groups. HO-1 immunoreactivity was induced in the CA1 pyramidal neurons in both IPC+sham-operated- and IPC+ischemia-operated groups. We also found that levels or immunoreactivities of superoxide anion, 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal were significantly decreased in the CA1 of both IPC+sham-operated- and IPC+ischemia-operated groups. Whereas, treatment with zinc protoporphyrin IX (a HO-1 inhibitor) into the IPC+ischemia-operated groups did not preserve the IPC-mediated increase of HO-1 and lost beneficial effects of IPC by inhibiting ischemia-induced DNA damage and lipid peroxidation. In brief, IPC protects CA1 pyramidal neurons from ischemic injury by upregulating HO-1, and we suggest that

Abbreviations: CO, carbon monoxide; CV, Cresyl violet; F-J B, Fluoro-Jade B; 4-HNE, 4-hydroxy-2-nonenal; HO-1, heme oxygenase-1; IPC, ischemic preconditioning; 8-OhdG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; ZnPP, zinc protoporphyrin IX.

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the enhancement of HO-1 expression by IPC may be a legitimate strategy for a therapeutic intervention of cerebral ischemic damage.

Introduction

Ischemic preconditioning (IPC) is a phenomenon in which a brief ischemic event can mobilize intrinsic protective mechanisms that increase tolerance against subsequent severe ischemic insult. Clinically, brief ischemic events induce tolerance by raising the threshold of tissue vulnerability, which is critical for neuroprotection [1]. IPC in the gerbil brain protects against neuronal damage following subsequent longer periods of ischemia [2]. This phenomenon has been termed "ischemic tolerance." Mechanisms of IPC-mediated tolerance are not precisely known; brain tissues with a high sensitivity against ischemia present the most promising targets for the therapeutic application of IPC [3–5].

Oxidative stress injury is the most crucial pathological link for neuronal injury. Reactive oxygen species (ROS) are produced in the course of normal metabolism [6]; the accumulation of ROS leads to oxidative stress, which promotes damage to carbohydrates, lipid, DNA and proteins [7,8], and neuronal death by oxidizing various cellular components, resulting in the "delayed neuronal death" in global cerebral ischemia [9].

Heme oxygenase (HO) belongs to the heat-shock protein family required for the conversion of heme into carbon monoxide (CO), iron, and biliverdin/bilirubin [10–12] and is popular for its cytoprotective functions such as its antioxidant, anti-inflammatory, anti-immune and antiapoptotic activities [13,14]. HO-1, which is an inducible isoform, is highly increased in response to a variety of cellular stresses and stimuli including ischemia [13]. It has been suggested that HO-1 expression and activity are increased by various preconditioning stimuli in the brain [15,16]. HO-1 exerts dual role, as it can be either protective or deleterious to the central nervous system, depending on expression levels of enzymes [17]. While its beneficial effects are related to a decrease in free heme concentration and further production of antioxidant compounds (biliverdin/bilirubin), massive HO-1 activation may cause toxic compound accumulation (CO and iron (Fe^{2+}) [18,19].

Various possible explanations exist for the neuroprotective effect of IPC against ischemic damage. IPC triggers multiple mechanisms including HO-1 induction that eventually dictates cellular responses to more severe injury [20,21]. However, the question of whether the expression of IPC-mediated HO-1 contributes to neuroprotection against ischemia still remains to be fully addressed. We hypothesize that IPC-mediated HO-1 expression represents an important defense against secondary injury after global cerebral ischemia by inhibiting DNA damage and lipid peroxidation. We, therefore, examined HO-1 immunoreactivity and its protein levels, which may be related to neuronal death in the hippocampus in control and IPC-induced gerbil brains induced by transient global cerebral ischemia.

Materials and methods

Experimental animals

We used male Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea. Gerbils were used at 6 months (bw, 65–75 g) of age. The animals were housed in a conventional state under adequate temperature (23 °C) and humidity (60%) control with a 12-h light/12-h dark cycle, and provided with free access to water and food. The procedures for animal handling and care adhered to guidelines that are in compliance with the current international laws and policies (*Guide for the Care and Use of Laboratory Animals,* The National Academies Press, 8th ed., 2011) and they were approved by the Institutional Animal Care and Use Committee (IACUC) at Kangwon University. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Induction of transient cerebral ischemia

The animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. A midline ventral incision was then made in the neck, and common carotid arteries were bilaterally isolated, freed of nerve fibers, and occluded using nontraumatic aneurysm clips (Yasargil FE 723K, Aesculap, Tuttlingen, Germany). The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope (HEINE K180, Heine Optotechnik, Herrsching, Germany). After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The restoration of blood flow (reperfusion) was observed directly using the ophthalmoscope. Body (rectal) temperature was maintained under free-regulating or normothermic (37 ± 0.5 °C) conditions with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and a thermometric blanket before, during, and after the surgery until the animals completely recovered from anesthesia. Thereafter, animals were kept in a thermal incubator (temperature, 23 °C; humidity, 60%) (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature until the animals were euthanized. The animals were divided into four groups: (1) sham-operated group ($n=7$ at each time point), the bilateral common carotid arteries were exposed, no ischemia was given (sham operation) in the animals; (2) ischemia-operated group ($n=7$ at each time point), the animals were given a 5 min lethal ischemic insult 24 h after sham operation (3) IPC plus(+) sham-operated group ($n=7$ at each time point), the animals were subjected to a 2 min sublethal ischemic insult; and (4) IPC+ischemia-operated group ($n=7$ at each time point), the animals were pretreated with a 2 min sublethal ischemia 1 day prior to a 5 min lethal ischemia. The animals in groups 2 and 4 were given recovery times of 12 h, 1 day, 2 days, and 5 days, because pyramidal neurons in the hippocampal CA1 region do not die until 3 days and begin to die 4 days after ischemia–reperfusion. This preconditioning paradigm has been proven to be very effective in protecting neurons from ischemic damage in this ischemic model [2].

Treatment with an inhibitor of HO-1

To examine the inhibition of HO-1 function on IPC effect, zinc protoporphyrin IX (ZnPP) (30 mg/kg, Sigma–Aldrich, St. Louis, MO), a specific inhibitor of HO-1, was intraperitoneally administered 3 times on IPC-operated animals: the first at 12 h before ischemia–reperfusion, the second 30 min after ischemia–reperfusion, and the third at 12 h after ischemia–reperfusion. The dose of ZnPP used in this study was selected according to the results of previous studies [22,23]. The ZnPP-treated animals ($n=7$ at each time point) were given recovery times of sham, 12 h, 1day, 2 days, and 5 days after ischemia–reperfusion.