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Effects of ischemic preconditioning on VEGF and pFlk-1 immunoreactivities in the gerbil ischemic hippocampus after transient cerebral ischemia

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ABSTRACT

Ischemia preconditioning (IPC) displays an important adaptation of the CNS to sub-lethal ischemia. In the present study, we examined the effect of IPC on immunoreactivities of VEGF-, and phospho-Flk-1 (pFlk-1) following transient cerebral ischemia in gerbils. The animals were randomly assigned to four 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. In the ischemia-operated-group, a significant loss of neurons was observed in the stratum pyramidale (SP) of the hippocampal CA1 region (CA1) alone 5 days after ischemia–reperfusion, however, in all the IPC + ischemia-operated-groups, pyramidal neurons in the SP were well protected. In immunohistochemical study, VEGF immunoreactivity in the ischemia-operated-group was increased in the SP at 1 day post-ischemia and decreased with time. Five days after ischemia–reperfusion, strong VEGF immunoreactivity was found in non-pyramidal cells, which were identified as pericytes, in the stratum oriens (SO) and radiatum (SR). In the IPC + sham-operated- and IPC + ischemia-operated-groups, VEGF immunoreactivity was significantly increased in the SP. pFlk-1 immunoreactivity in the sham-operated- and ischemia-operated-groups was hardly found in the SP, and, from 2 days post-ischemia, pFlk-1 immunoreactivity was strongly increased in non-pyramidal cells, which were identified as pericytes. In the IPC + sham-operated-group, pFlk-1 immunoreactivity was significantly increased in both pyramidal and non-pyramidal cells; in the IPC + ischemia-operated-groups, the similar pattern of VEGF immunoreactivity was found in the ischemic CA1, although the VEGF immunoreactivity was strong in non-pyramidal cells at 5 days post-ischemia. In brief, our findings show that IPC dramatically augmented the induction of VEGF and pFlk-1 immunoreactivity in the pyramidal cells of the CA1 after ischemia–reperfusion, and these findings suggest that the increases of VEGF and Flk-1 expressions may be necessary for neurons to survive from transient ischemic damage.

1. Introduction

When the blood supply to the brain is disrupted, the tissue deprivation of oxygen and glucose occurs and it may give irreversible brain damage [1,2]. A brief period of global brain ischemia causes cell damage/death in vulnerable hippocampal CA1 region a few days after reperfusion, and is referred to as “delayed neuronal death” [3]. Many mechanisms related to ischemia-induced delayed neuronal death, including reactive oxygen species, oxidative stress and DNA damage, have been suggested [4–6]. However, the mechanisms underlying them have not been exactly elucidated yet. Ischemia preconditioning (IPC) represents an important adaptation of the central nervous system (CNS) to sub-lethal ischemia, which results in increased ischemia tolerance of the CNS to a subsequent longer or lethal period of

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ischemia [7,8]. IPC induces the expression of a diverse family of genes involved in cytoprotection, which, in turn, encodes proteins that serve to enhance brain resistance to ischemia [9,10]. This phenomenon has been termed "ischemic tolerance", and its basic mechanisms underlying ischemic tolerance are not fully understood yet [11].

Vascular endothelial growth factor (VEGF) is well known as an angiogenic and vascular permeability factor [12,13], and plays a regulatory role in the nervous system [14,15]. In the developing nervous system, VEGF acts as a neurotrophic factor, regulates axonal outgrowth and increases neuronal survival [16,17]. On the other hand, VEGF protects hippocampal neurons from glutamate toxicity in vitro [18]. Moreover, it has been well known that VEGF improves cognitive deficits and neuronal protection against ischemic injury, and it is suggested that VEGF may participate in the brain's endogenous response to ischemic injury [19–22]. In addition to VEGF itself, two main classes of receptors for the VEGF have been identified: the tyrosine kinase and nontyrosine kinase receptors. The former contains three structurally related receptors, VEGFR-A (Flt-1), VEGFR-B (Flk-1), and VEGFR-C. The nontyrosine receptors consist of neuropilin-1 (NP-1) and neuropilin-2 (NP-2). Flk-1 is a major mediator of angiogenic and permeability-enhancing effects of VEGF [23,24], and its expression is up-regulated in neurons, glial cells, and endothelial cells by focal cerebral ischemia [25–27] and global cerebral ischemia [28]. Furthermore, we recently reported that VEGF, which was expressed in the gerbil hippocampus, might be related with neuronal loss after transient global cerebral ischemia, and its expression was different according to aging [29].

However, little is known regarding expression patterns of VEGF and Flk-1 and phenotypes of cells expressing VEGF and Flk-1 in the brain of IPC-mediated animals induced by transient cerebral ischemia. This study, therefore, was performed to investigate temporal changes and cellular localization of VEGF and phospho-Flk-1 (pFlk-1) in the gerbil hippocampus following transient global cerebral ischemia. We also examined changes in VEGF and pFlk-1 expressions in the brain of IPC-mediated gerbils.

2. Materials and methods

2.1. Experimental animals

We used the male Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea. Gerbils were used at 6 months (B.W., 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 were provided with free access to food and water. 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.

2.2. Induction of transient cerebral ischemia and animal groups

Transient cerebral ischemia was developed following our previous method. [6]. The experimental animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Under an operating microscope, ischemia was induced by occluding the common carotid arteries with non-traumatic aneurysm clips (Yasargil FE 723 K, Aesculap, Tuttlingen, Germany) for 2 min or 5 min. The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope (HEINE K 180®, Heine Optotechnik, Herrsching, Germany). The body (rectal) temperature was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and maintained under free-regulating or the normothermic (37 ± 0.5 °C) conditions using a thermometric blanket for the surgery. Thereafter, animals were kept on the thermal incubator (temperature, 23 °C; humidity, 60%) (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature of animals 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 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 at protecting neurons against ischemia in this ischemic model [30].

2.3. Tissue processing for histology

All of the animals were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30 μm coronal sections, and they were then collected into six-well plates containing PBS.

2.4. Cresyl violet (CV) staining

To examine the neuronal death in the hippocampal CA1 region in each group using CV staining, the sections were mounted on gelatin-coated microscopy slides. Cresyl violet acetate (Sigma–Aldrich, St. Louis, MO) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid was added to this solution. The sections were stained and dehydrated by immersing in serial ethanol baths, and they were then mounted with Canada balsam (Kanto chemical, Tokyo, Japan).

2.5. Neuronal nuclei immunohistochemistry

To examine the neuronal changes in the hippocampal CA1 region after transient cerebral ischemia using anti-neuronal nuclei (NeuN, a marker for neurons), the sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. The sections were next incubated with diluted mouse anti-NeuN (a neuron-specific soluble nuclear antigen) (diluted 1:1000, Chemicon International, Temecula, CA) overnight at 4 °C. Thereafter the tissues were exposed to biotinylated horse anti-mouse IgG and streptavidin peroxidase complex (Vector, Burlingame, CA). And they were visualized with 3,3′-diaminobenzidine in 0.1 M Tris–HCl buffer and mounted on the gelatin-coated slides. After dehydration the sections were mounted with Canada balsam (Kanto chemical).

2.6. Fluoro-Jade B (F-J B) histofluorescence

To examine neuronal death in the CA1 region at each time point after ischemia using F-J B (a high affinity fluorescent marker for the localization of neuronal degeneration) histofluorescence [31], the sections were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol, and followed in 70% alcohol. They were then