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Impact of hyperthermia before and during ischemia–reperfusion on neuronal damage and gliosis in the gerbil hippocampus induced by transient cerebral ischemia

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ABSTRACT

Hyperthermia can exacerbate the brain damage produced by ischemia. In the present study, we investigated the effects of hyperthermia before and during ischemia–reperfusion on neuronal damage and glial changes in the gerbil hippocampus following transient cerebral ischemia using cresyl violet staining, NeuN immunohistochemistry and Fluoro-Jade B histofluorescence staining. The animals were randomly assigned to 4 groups: (1) sham-operated animals with normothermia (normothermia + sham group); (2) ischemia-operated animals with normothermia (normothermia + ischemia group); (3) sham-operated animals with hyperthermia (hyperthermia + sham group); and (4) ischemia-operated animals with hyperthermia (hyperthermia + ischemia group). Hyperthermia (39.5 ± 0.2 °C) was induced by exposing the gerbils to a heating pad connected to a rectal thermistor for 30 min before and during ischemia–reperfusion. In the normothermia + ischemia groups, a significant delayed neuronal death was observed in the stratum pyramidale (SP) of the hippocampal CA1 region (CA1) 5 days after ischemia–reperfusion. In the hyperthermia + ischemia groups, neuronal death in the SP of the CA1 occurred at 1 day post-ischemia, and neuronal death was observed in the SP of the CA2/3 region at 2 days post-ischemia. In addition, we examined activations of astrocytes and microglia using immunohistochemistry for anti-glial fibrillary acidic protein (GFAP) and anti-ionized calcium-binding adapter molecule 1 (Iba-1). GFAP-positive astrocytes and Iba-1-positive microglia in the ischemic hippocampus were activated much earlier and much more accelerated in the hyperthermia + ischemia groups than those in the normothermia + ischemia groups. Based on our findings, we suggest that an experimentally hyperthermic pre-condition before cerebral ischemic insult produces more extensive neuronal damage and glial activation in the ischemic hippocampus.

1. Introduction

The brain is very vulnerable to ischemia because of its high metabolic rate, low oxygen stores and an insufficient reserve of high-energy carbohydrates compared with the other tissues [1]. During global forebrain ischemia, the reduction of blood supply to the brain triggers a number of neuro-pathophysiological processes that result in irreversible neuronal damage in sensitive regions, such as the hippocampus [2]. In the hippocampus, the vulnerability differs from each hippocampal subregion: the hippocampal CA1 region is the most vulnerable to transient forebrain ischemia, whereas the CA3 region is the most resistant to the ischemia [3]. This unique process in the CA1 region is termed "delayed neuronal death", which occurs from 4 days after 5 min of transient forebrain ischemia [2,4]. The delayed neuronal death is due to diverse cellular changes, such as DNA damage and oxidative stress

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following ischemia–reperfusion [5,6]. However, the precise mechanisms of the delayed neuronal death remain unclear. The Mongolian gerbil has been used as a good animal model to investigate mechanisms of selective delayed neuronal death following transient global forebrain ischemia [7–9], because about 90% of the gerbils lack the communicating vessels between the carotid and vertebral circulations. Thus, the bilateral occlusion of the common carotid arteries essentially and completely eliminates blood flow to the forebrain while completely sparing the vegetative centers of the brain stem.

Body temperature is a major factor in neuronal survival/death after cerebral ischemia; hypothermia is neuroprotective and hyperthermia is damaging [10–12]. Hyperthermia (>38 °C) has been included as an independent prognostic marker for the prediction of mortality and functional outcome after an acute ischemic stroke [13,14]. Preclinical studies have provided several evidences for the harmful effects of elevated body temperature after post-ischemia on different animal models of ischemia, such as global forebrain ischemia [15], focal permanent ischemia [16] and focal transient ischemia [17,18]. Clinical data have confirmed that mild hyperthermia in ischemic stroke patients can enlarge infarct size and worsen the outcome of ischemic stroke [19,20]. The brain temperature can reach above 40.5 °C after transient forebrain ischemia. At this temperature, the ability of the hypothalamus becomes compromised to coordinate thermoregulation and there can be a further increase in brain temperature, low blood pressure, an increase in intracerebral pressure, monoamine overload, and multi-organ dysfunction [21,22]. Although it is well known that a rise in body temperature after experimentally induced transient forebrain ischemia produces more extensive brain damage [23], studies regarding neuronal damage/death in the hippocampus according to hyperthermic condition before ischemic insults are not reported.

Therefore, in the present study, we examined whether hyperthermic condition before an ischemic insult is associated with neuronal damages and glial changes in the hippocampus of the gerbil, which is a good animal model of ischemic stroke [24–26], following transient forebrain ischemia.

2. Materials and methods

2.1. Experimental animals

Male Mongolian gerbils (*Meriones unguiculatus*) were 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 ± 0.2 °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 forebrain ischemia

Experimental animals ($n = 7$ at each time point per group) were divided into four groups: (1) sham-operated animals with normothermia (normothermia + sham group); (2) ischemia-operated animals with normothermia (normothermia + ischemia group); (3) sham-operated animals with hyperthermia (hyperthermia + sham group); and (4) ischemia-operated animals with hyperthermia (hyperthermia + ischemia group). Hyperthermia was induced by exposing the gerbils to a heating pad connected to a rectal thermistor while the animals were under anesthesia until their rectal temperature was elevated to 39.5 ± 0.2 °C, and the animals were maintained at this temperature for 30 min before and during ischemia–reperfusion.

The animals of all the groups were anesthetized with a mixture of 2.5% isoflurane (Baxter, Deerfield, IL) in 33% oxygen plus 67% nitrous oxide gas. Bilateral common carotid arteries were isolated and occluded using non-traumatic aneurysm clips (Yasargil FE 723K, Aesculap, Tuttlingen, Germany). Bilateral common carotid arteries were occluded using non-traumatic aneurysm clips for 5 min. The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope (HEINE K180®, Heine Optotechnik, Herrsching, Germany). During the surgery, the animals of the normothermia and hyperthermia groups were kept on the heating pad at 37 ± 0.2 °C and 39.5 ± 0.2 °C, respectively. Thereafter, the animals were kept in the thermal incubator (temperature, 23 °C; humidity, 60%) (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature on the normothermic level until they were euthanized. Sham-operated animals were exposed to similar surgery without carotid artery occlusion. The animals in each group 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 from 4 days after ischemia–reperfusion.

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 (CM1900 UV, 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 neuronal damage/death in the hippocampus at each time point after transient forebrain ischemia 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. NeuN immunohistochemistry

To investigate the neuronal damage/death in the hippocampus at each time point after transient forebrain 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 (diluted 1:1000, Chemicon International, Temecula, CA) overnight at 4 °C. Thereafter the tissues were exposed to biotinylated goat anti-mouse IgG (Vector, Burlingame, CA) and streptavidin peroxidase complex (diluted 1:200, Vector). They were then visualized by staining with 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M Tris–HCl buffer (pH 7.2) and mounted on gelatin-coated slides. After dehydration, the sections were mounted with Canada balsam (Kanto Chemical).

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

To confirm the neuronal death in the hippocampus at each time point after transient forebrain ischemia using F-J B (a high affinity fluorescent marker for the localization of neuronal degeneration)