Research report

Hypothermia inhibits ischemia-induced efflux of amino acids and neuronal damage in the hippocampus of aged rats

Hiroaki Ooboshi a,*, Setsuro Ibayashi a, Kentaro Takano a, Seizo Sadoshima a, Akira Kondo b, Hideyuki Uchimura b, Masatoshi Fujishima a

a Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan
b Center for Emotional and Behavioral Disorders, Hizen National Mental Hospital, Saga, Japan

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Abstract

Brain hypothermia has been reported to protect against ischemic damages in adult animals. Our goal in this study was to examine whether brain hypothermia attenuates ischemic neuronal damages in the hippocampus of aged animals. We also determined effects of hypothermia on ischemia-induced releases of amino acids in the hippocampus. Temperature in the hippocampus of aged rats (19–23 months) was maintained at 36 °C (normothermia), 33 °C (mild hypothermia) or 30 °C (moderately hypothermia) using a thermoregulator during 20 min of transient forebrain ischemia. Cerebral ischemia increased extracellular concentrations of glutamate and aspartate by 6- and 5-fold, respectively, in the normothermic group. Mild and moderate hypothermia, however, markedly inhibited the rise of these amino acids to less than 2-fold. Elevation of extracellular taurine, a putative inhibitory amino acid, was 16-fold in the normothermic rats. Mild hypothermia attenuated ischemia-induced increase in taurine (10-fold), and moderate hypothermia inhibited the increase. Ischemic damages, evaluated by histopathological grading of hippocampal CA1 area 7 days after ischemia, was significantly ameliorated in the mild (1.3±0.5, mean±S.E.M.) and moderate hypothermic rats (0.8±0.3) compared with the normothermic ones (3.4±0.4). These results suggest that brain hypothermia protects against ischemic neuronal damages even in the aged animals, and the protection is associated with inhibition of excessive effuxes of both excitatory and inhibitory amino acids.

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Topic: Ischemia

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1. Introduction

Stroke is the leading cause of death in the Japanese elderly [34], and aging is one of the major risk factors for cerebrovascular disease. Although the importance of studies that use aged animals to examine brain ischemia has been claimed [25], such experiments are limited [12,14]. We have developed the experimental model for brain ischemia using aged spontaneous hypertensive rats (SHR), more relevant models for human stroke. Recently we reported that the hippocampus of aged SHR was more susceptible to transient cerebral ischemia than adult SHR [37].

Brain hypothermia is reported to protect neuronal cells against ischemic insults in the adult and infant animals [6,4,23,36]. However, effects of hypothermia on the ischemic damage in the aged animals have not been well clarified. Our first goal in this study was to examine whether brain hypothermia attenuates the ischemia-induced neuronal damage in the hippocampus of aged SHR.

Increases in extracellular excitatory amino acids have been reported to play a pivotal role in ischemic neuronal damages [10,31,32,34]. Extracellular inhibitory amino acids also increase during cerebral ischemia [16,18,38], and the vulnerability of hippocampus in the aged rats is suggested to be related with changes in inhibitory amino
24

Ooboshi et al. / Brain Research 884 (2000) 23–30

Therefore, the second goal in this study was to determine effects of hypothermia on the effluxes of excitatory and inhibitory amino acids in the aged rats. We monitored the level of extracellular amino acids using a microdialysis method, and compared with the severity of histopathological damages. Regional brain temperature in the hippocampus was satisfactorily controlled by the specific thermoregulator [19].

2. Materials and methods

2.1. Animals

Nineteen aged female SHR, 19–23 months old and weighed 195–215 g, were used in this study. Rats were maintained in Animal Center, Kyushu University under specific pathogen-free conditions, and fed stock chow diet and tap water ad libitum. All experimental procedures were performed in accordance with the Physiological Society of Japan Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences. Female SHR were chosen because of their low mortality rate (under 30% at 20 months), while a half of male SHR expired between 15 and 20 months.

2.2. Surgery

Cerebral ischemia was produced by bilateral carotid artery occlusion [13]. Briefly, each rat was anesthetized with amobarbital (100 mg/kg i.p.) and breathed room air spontaneously. Both femoral arteries were cannulated, one for sampling blood to measure arterial blood gases and pH with an IL meter Model 1304 (Instrumentation Laboratory Inc., Lexington, MA, USA) and the other for continuous recording of blood pressure. Bilateral common carotid artery was exposed through a ventral incision in the neck, carefully separated from the vagosympathetic trunks, and loosely encircled with sutures for later retraction.

2.3. Microdialysis

Concentrations of extracellular amino acids and cerebral blood flow (CBF) in the hippocampal CA1 subfield were simultaneously determined using a microdialysis technique and a hydrogen clearance method, respectively [29]. Each rat was fixed in a head holder, and a small burr hole was made in the parietal region. A dialysis probe with 1-mm membrane (CMA-10: Carnegie Medicine, Stockholm, Sweden) and a Teflon-coated electrode with micro-thermocouple (300 μm in diameter, 1-mm portion at its tip uncoated) for measurement of both CBF and brain temperature were placed stereotaxically in the right hippocampal CA1; 4.3 mm posterior and 1.5 mm lateral to the bregma and 3.0 mm in depth from the surface of the brain. Ringer’s solution (147 mM Na+, 2.3 mM Ca2+, 4 mM K+, 155.5 mM Cl−, pH 7.4) was infused in the hippocampus through the dialysis probe at a rate of 4.0 μl/min with a syringe pump (Eicom Co., Kyoto, Japan). The perfusate was collected every 10 min into a plastic tube and stored at −80°C for the later measurement of amino acids.

2.4. Brain hypothermia

Brain temperature was modulated by a selective brain thermoregulator (metallic plate brain cooling device) which we developed (model BTC-100, Unique Medical Co., Tokyo, Japan). Briefly, an aluminum metallic plate (22 mm×16 mm×1 mm) consisted of two thermomodules (one for heating and the other for cooling using a water-circulating system) was placed on the surface of rat’s scalp, and the thermocouple was inserted through an elliptical center hole (8 mm×6 mm) in the plate. The device with a continuous-monitoring system can quickly and precisely adjust the cerebral cortex at desired brain temperature [19]. Following a resting period of 90 min, the baseline CBF and arterial blood pressure were determined with hippocampal temperature of 36°C using the thermoregulator. Then, the temperature of the hippocampus was adjusted to 30°C (moderate hypothermia, n=6) or 33°C (mild hypothermia, n=6) or remained 36°C (normothermia, n=7) until 80 min after brain ischemia. Rectal temperature was maintained at 37°C using a heat pad throughout the experiment. Both carotid arteries were ligated for 20 min, followed by 80 min of recirculation. CBF was determined at every 10 min of ischemia and recirculation. Arterial blood gases, pH and hematocrit were measured at the resting period, 20 min of ischemia and 80 min of recirculation.

2.5. HPLC analysis

Concentrations of amino acids were measured using HPLC combined with fluorescent detection after precolumn derivatization. Each sample was automatically mixed with α-phthalaldehyde and 2-mercaptoethanol for 2 min, and then injected into the HPLC system, which consisted of an Eicom pump (Eicom Co., Kyoto, Japan) at a flow rate of 1.0 ml/min, a reverse phase column (Eicomac MA-5 ODS, 4.6×250 mm, Eicom Co., Kyoto, Japan) and a fluorescent detector (Shimadzu Co., Tokyo, Japan). The mobile phase was 0.1 M sodium phosphate (pH 6.0) containing 30% (vol/vol) methanol. Concentrations of glutamate, aspartate, glycine, taurine, GABA and alanine were determined by comparison of standard solution.

2.6. Histological examination

After 80 min of recirculation, the dialysis probe and electrodes were withdrawn, and bone wax was pasted on
the hole in the skull. The femoral arteries were ligated, and incisions in the head, neck and leg were sutured. Each rat was brought back to the cage and freely fed water and food. Seven days after forebrain ischemia, five rats in the 36°C groups and four rats in each hypothermic group were anesthetized with amobarbital (100 mg/kg i.p.). Brains were transcardially perfused with 4% paraformaldehyde in 1/15 M phosphate buffer (pH 7.3) after a brief wash-out period with heparinized saline. Each brain was removed and fixed in 4% neutral formaldehyde for 7 days. Paraffin sections were taken at the level of the hippocampus in each rat and were stained with hematoxylin and eosin. Ischemic neuronal damage of the hippocampal CA1 subfield in each hemisphere was graded from 0 to 3 (3, majority of neurons damaged; 2, many neurons damaged; 1, a few neurons damaged; or 0, normal) by a neuropathologist (A.K.) without knowledge of the experimental conditions, and the summed value of both hemispheres was regarded as ischemic score for each animal.

2.7. Statistical analysis

All values were presented as mean±S.E.M. The statistical differences among and within groups for physiological parameter, CBF and concentrations of amino acids were analyzed by two-way repeated analysis of variance followed by Dunnett’s t-test. Ischemic scores for neuronal damages were compared among groups by nonparametric Kruskal–Wallis’ h-test followed by Dunnett’s t-test.

3. Results

3.1. Physiological parameters

Table 1 depicts physiological parameters at the resting period, 10 min of ischemia, and 80 min of recirculation. Mean arterial blood pressure increased by approximately 30 mmHg during carotid artery occlusion, and recovered to the resting values during recirculation. Each rat developed respiratory alkalosis during ischemia. There was no significant difference in any physiological parameters among the groups. Changes in CBF to the hippocampus are present in Fig. 1. CBF before ischemia was 46.3±2.2 ml/100 g/min, 44.4±5.0 and 47.7±4.7 in the 36, 33 and 30°C group, respectively. Brain hypothermia for 20 min did not alter the blood flow. Bilateral carotid artery occlusion for 20 min reduced CBF to less than 12 ml/100 g/min in all groups. CBF increased from 85 to 160% of the resting value immediately after recirculation, followed by mild hypoperfusion (60–80% of the resting value) in all groups. There was no significant difference in hippocampal blood flow during and after brain ischemia among the groups (F2,16=0.89).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean arterial blood pressure (MABP), arterial gases, pH and hematocrit at resting period, 10 min of ischemia and 80 min of recirculation</th>
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<tr>
<td></td>
<td>At rest</td>
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<tr>
<td>MABP (mmHg)</td>
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<tr>
<td>36°C</td>
<td>165±7</td>
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<td>33°C</td>
<td>165±8</td>
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<tr>
<td>30°C</td>
<td>169±3</td>
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<tr>
<td>PaCO₂ (mmHg)</td>
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<tr>
<td>36°C</td>
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<tr>
<td>33°C</td>
<td>43.5±1.7</td>
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<tr>
<td>30°C</td>
<td>44.4±1.2</td>
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<tr>
<td>PaO₂ (mmHg)</td>
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<td>pH</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>33°C</td>
<td>40.2±1.9</td>
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<tr>
<td>30°C</td>
<td>45.1±1.8</td>
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Values are means±S.E.M. (n=6–7). Paired data were compared with control values by analysis of variance and Dunnett’s t-test (* P<0.05, ** P<0.01). No significant difference was found among the groups. (a) P<0.05, (b) P<0.01 compared with the basal value.
though concentrations of GABA were not detectable at the resting periods, the basal values of the other amino acids did not differ among the three groups, and changes in concentrations of amino acids were shown as percentages of basal values.

3.3. Excitatory amino acids

Concentrations of glutamate before ischemia were not altered by brain hypothermia. The extracellular glutamate significantly increased approximately 6-fold \( (P<0.01) \) during bilateral carotid artery occlusion in the 36°C group (Fig. 2). Concentrations of glutamate returned to the basal level after 20 min of recirculation. Ischemia-induced elevation of glutamate was markedly attenuated \( (F_{2,16}=6.17; P<0.02) \) to less than 2-fold the basal values in both hypothermia groups. The time course of extracellular aspartate was similar to glutamate (Fig. 2), showing 5-fold increases by ischemia in the normothermic group, but no significant elevation in hypothermic groups.

3.4. Inhibitory amino acids

Twenty min after induction of hypothermia, concentrations of taurine were significantly reduced to 80% of the basal value. In the 36°C group, taurine increased to a greater extent (16-fold) than glutamate and aspartate during and soon after cerebral ischemia (Fig. 3). On the other hand, changes in taurine levels in the 30°C group were only 3-fold and not significant compared with basal levels. The elevation of taurine in the 33°C group, however, was still marked (10-fold, \( P<0.05 \)). Concentrations of GABA increased to 171.0±32.3 nM during ischemia in five of seven rats in the 36°C group. In contrast, only one of six rats in each hypothermic group showed detectable GABA concentrations (33°C group, 88.5 nM; 30°C, 58.8 nM).

3.5. Other amino acids

The ischemia-induced increase of glycine was relatively small (2-fold) but significant in the normothermic group.

Fig. 2. Changes in concentrations of glutamate, aspartate and glycine in the dialysate in the 36°C (n=7), 33°C (n=6) and 30°C (n=6) groups. Data are expressed as mean±S.E.M. of percentages of the resting value. Differences among the groups were significant in the concentrations of glutamate \( (F_{2,16}=6.17; 0.02) \), aspartate \( (F_{2,16}=4.55; P<0.05) \) and glycine \( (F_{2,16}=13.0; P<0.001) \) by two-way repeated measured analysis of variance. * \( P<0.05 \), ** \( P<0.01 \), difference of 33°C from 36°C, * \( P<0.05 \), ** \( P<0.01 \), difference of 30°C from 36°C by one-way analysis of variance and Dunnett’s \( t \)-test. The ischemia-induced increases in glutamate, aspartate and glycine were significant only in the 36°C group. (a) \( P<0.01 \) compared with the basal value by one-way analysis of variance and Dunnett’s \( t \)-test.
Fig. 3. Changes in concentrations of taurine and alanine in dialysate in the 36°C (n=7), 33°C (n=6) and 30°C (n=6) groups. Data are expressed as mean±S.E.M. of percentages of the resting value. Differences among the groups were significant in the concentrations of taurine ($F_{2,16}=5.59; P<0.02$) and alanine ($F_{2,16}=12.2; P<0.001$) by two-way repeated measured analysis of variance. * $P<0.05$, ** $P<0.01$, difference of 33°C from 36°C, * $P<0.05$, ** $P<0.01$, difference of 30°C from 36°C by one-way analysis of variance and Dunnett’s t-test. The ischemia-induced increases in taurine and alanine were significant in the 36°C and 33°C groups. (b) $P<0.05$, (a) $P<0.01$ compared with the basal value by one-way analysis of variance and Dunnett’s t-test.

Fig. 4. Photomicrographs of the CA1 subfield of the hippocampus of SHR 7 days after transient cerebral ischemia. In the 36°C rat (a), majority of pyramidal cells revealed shrunken cytoplasm and picnotic nuclei associated with perinuclear vacuolation. Nuclei of glial cells were also increased in number. There were a few degenerative pyramidal cells in the 33°C rat (b). No apparent ischemic damage was observed in the 30°C rat (c). Paraffin section with hematoxylin and eosin stain. Magnification, $\times300$. 
The extracellular glycine decreased by 20% at 20 min after induction of hypothermia, which lasted during ischemia and recirculation. Concentrations of alanine in the normothermic rats significantly elevated 3-fold during ischemia, and remained at high values during recirculation (Fig. 3). The alanine level was reduced in rats with hypothermia during and after cerebral ischemia ($F_{2,16} = 12.2; \ P < 0.001$).

### 3.6. Histological examination

Photomicrographs of the CA1 subfield of the hippocampus 7 days after cerebral ischemia are shown in Fig. 4. In rats of the normothermic group, most pyramidal cells revealed shrunken cytoplasm with acidophilic changes and pyknotic nuclei associated with perinuclear vacuolation. Glial cells also increased in number. In contrast, degenerative pyramidal cells in the 33°C group were scattered, and ischemic damages were markedly attenuated in the 30°C group. Scores for ischemic damages (Fig. 5) were significantly attenuated in both mild (1.3 ± 0.5) and moderately (0.8 ± 0.3) hypothermic groups as compared with the normothermic group (3.4 ± 0.4).

### 4. Discussion

In this study, we demonstrated that ischemic neuronal damages in the hippocampus of aged SHR were markedly protected by mild and moderate reduction of brain temperature. Furthermore, the protection was associated with inhibition of ischemia-induced effluxes of excitatory (glutamate, aspartate) and inhibitory (taurine, GABA) amino acids in the hippocampus, which was for the first time demonstrated in the aged ischemic model.

Because most of ischemic stroke occur in the elderly populations [35] and the age-related vulnerability to ischemia are reported [12,37], it is important to examine the pathophysiology of brain ischemia and to explore effective treatment with aged models [23]. Those studies are, however, limited [11,12,37]. Therefore, our study using the aged hypertensive animals would provide useful information for treatment of brain ischemia in aged populations.

Because CBF and other physiological parameters were similar among normothermic and hypothermic animals in our study, factors other than circulation are suggested to contribute to the protection of hippocampal neurons. One possible mechanism is the altered effluxes of excitatory amino acids, because ischemia-induced effluxes of glutamate and aspartate are regarded to play crucial roles in the development of ischemic neuronal damages in the adult animals [10,31,32,34]. Interestingly, our results revealed complete inhibition of effluxes of glutamate and aspartate in the hippocampus by mild decreases of brain temperature (by 3°C). In the previous reports with adult animals [22,24], mild brain hypothermia (approximately 3°C reduction) provided moderate reductions of the ischemia-induced effluxes in the hippocampus. Therefore, mild brain hypothermia may be more effective in the aged animals to inhibit effluxes of excitatory amino acids, thereby leading to effective protection of hippocampal neurons.

On the other hand, inhibitory amino acids, i.e., GABA and taurine, increase substantially in the extracellular space during ischemia [14,16], and are suggested to protect neuronal cells against ischemic damages [1,20,33]. The protective effects of inhibitory amino acids are reported to be mediated by hyperpolarization via chloride channels [5,17,28]. Although recent studies suggest that the balance of excitatory and inhibitory amino acids is related to the selective vulnerability of the hippocampus in the adult models [15,21], we have shown that the imbalance is involved in the age-related vulnerability of hippocampus to ischemia [30]. Therefore, the significant elevation (10-fold) of taurine in the mild hypothermia (33°C) may have contributed to neuronal protection.

Few reports are available regarding mechanisms for attenuation of ischemia-induced effluxes of neurotransmitters by brain hypothermia. It is reported that hypothermia prevents translocation of protein kinase C in cerebral ischemia [8]. Activation of synaptic enzymes such as protein kinase C or calcium-calmodulin kinase II facilitates the release of transmitters [10,27], and protein kinase C is involved in ischemia-induced transmitter release [26]. Therefore, preventing overactivation of presynaptic enzymes may lead to attenuation of massive releases of neurotransmitters. Another possibility is preservation of uptake mechanisms of amino acids. The reversed operation of uptake mechanisms in neurons and
glia is suggested to be the major factor for effluxes of amino acids during brain ischemia, and the uptake carrier is driven by the transmembrane gradients for Na\(^+\)-K\(^+\) [2]. Because hypothermia attenuates ischemic depolarization [9], preservation of ionic gradients by hypothermia may attenuate the reversed operation of the uptake mechanism.

In conclusion, mild brain hypothermia markedly inhibited the ischemia-induced effluxes of both excitatory and inhibitory amino acids, and ameliorated ischemic neuronal damages in the hippocampus of aged SHR. Mild brain hypothermia may lead to one of the useful treatments against ischemic damages in the aged model.

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