Original

Measurement of cerebral reactive hyperemia at the initial post-ischemia reperfusion stage under normothermia and moderate hypothermia in rats

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Abstract: Inhibition of the initial events occurring immediately after ischemia-reperfusion seems to be beneficial for reducing the extent of subsequent chronic neuronal cell injury. We investigated the effects of moderate hypothermia (32°C) commencing 30 min before ischemia on reactive hyperemia by measuring cerebral blood flow (CBF) with a laser-Doppler flowmeter at the initial ischemia-reperfusion stage (60 min) following 10 min of global cerebral ischemia in rats. In normothermia, CBF was increased to approximately 240% and decreased thereafter, although it remained at approximately 150% after 60 min of ischemia-reperfusion. In contrast, hypothermia increased CBF to more than 270% after ischemia-reperfusion, then recovered to the basal level within 30 min. The period of reactive hyperemia under normothermia tended to be shortened by pre-administration of an NMDA antagonist, in a manner similar to hypothermia. Furthermore, hypothermia inhibited the presence of cells with caspase-3-like immunoreactivity in the hippocampal CA1 sector after 8 h of ischemia-reperfusion. Our findings indicate that hypothermia tends to shorten the period of reactive hyperemia during the initial ischemia-reperfusion stage. This phenomenon may be partly associated with activation of NMDA receptors and a beneficial effect of hypothermia in resisting progression of the neurotoxic cascade in the first 8 h after ischemia-reperfusion. (J Oral Sci 51, 615-621, 2009)

Keywords: caspase-3; hypothermia; ischemia-reperfusion; reactive hyperemia.

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Introduction

It has been become evident that several critical pathophysiological events occur during the initial stage of post-ischemia reperfusion that seem to be closely related to the progression of subsequent neuronal cell injury (1,2). In particular, reactive hyperemia plays a crucial role in progressive neuronal cell injury in the initial stage after ischemia-reperfusion (3-7). There is now strong evidence to suggest that moderate hypothermia is effective and established treatment for suppressing the exacerbation of neuronal cell injury caused by ischemia-reperfusion (2,8-11). Recently, it was suggested that the neuroprotective effects of hypothermia might be significantly increased when initiated immediately (within several minutes) after the occurrence of ischemic injury, and was proposed to be a potentially beneficial clinical strategy (12). In fact, our

group had previously reported that moderate intra-ischemic hypothermia markedly enhanced post-ischemic extra-cellular glutamate ([glu]e) re-uptake during the initial ischemia-reperfusion stage (13). In the present study, using a laser Doppler flowmeter, we determined the characteristic profiles of cerebral blood flow (CBF) in the very early stage (within several minutes) of post-ischemic reactive hyperemia caused by moderate hypothermia in rats. In addition, we carried out an immunohistochemical study to investigate active caspase-3, as a major factor participating in the initial stage of the apoptotic cascade (14,15) following ischemia-reperfusion in the nuclei of hippocampal neurons in the CA1 sector after 8 h.

Materials and Methods

Every effort was made to minimize animal suffering and reduce the number of animals used in this study. The experiments were performed under the authority of, and according to the guidelines established by the Ethics Committee of Kanagawa Dental College.

Reagents

Urethane was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A non-competitive antagonist of the NMDA receptor, (+)methyl-10,11-dihydro-5*H*-dibenzo [a,d]cyclohepten-5, 10-imine, dizocilpine (MK-801), was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Experimental procedures

Male Sprague-Dawley rats (275-350 g) were allowed food and water *ad libitum*, and used in the experiments. The animals were anesthetized with urethane (1.25 g/kg,

Fig. 1 Schematic diagram showing the methods used for maintaining temporal muscle temperature in each experimental group.

i.p.), tracheotomized, then intubated orotracheally and artificially ventilated with room air employing a small-animal ventilator (Rodent Ventilator, Ugo Basile, Rome, Italy). Mean arterial blood pressure (MABP) was monitored from the femoral artery. The head was fixed in a stereotactic frame, and a laser-Doppler flowmeter (ALF21RD, Advance Co. Ltd., Tokyo, Japan) probe with an outer diameter of 0.5 mm was placed on top of the skull surface and covered with aluminum foil as protection from overhead light interference (16). Temporal muscle temperature was monitored as an indicator of brain temperature by inserting a sensor probe into the left side to avoid direct surgical damage to the brain (17-19).

As shown in Fig. 1, in rats under normothermic ($n = 7$) and hypothermic ($n = 7$) conditions, commencing 30 min before induction of ischemia, pre- and intra-ischemic temporal muscle temperatures were maintained at 37 ± 0.5°C and 32 ± 0.5°C, respectively. Ten minutes of transient global cerebral ischemia was induced by occlusion of the bilateral carotid arteries and hemorrhagic hypotension (19-22). CBF was monitored with a laser Doppler flowmeter in accordance with previous descriptions (19-23). After ischemia had been terminated, temporal muscle temperature was allowed to recover immediately to 37.0 ± 0.5°C and maintained at that level throughout the remainder of the experiment (19,22). Target temperatures were obtained using an overhead light and/or small fan with a cooling cold spray via the top of the brain surface. Rectal temperature was maintained at 37.0 ± 0.5°C with a heating pad throughout the experiment.

CBF and MABP were both monitored continuously throughout the experiment, and their values were recorded every 0.25 s. The pre-ischemic CBF level (average value after 30 min at the stable pre-ischemic level) was defined as 100%. MK-801 was dissolved in saline and administered via an intraperitoneal injection (3 mg/kg, $n = 7$) at 30 min prior to induction of ischemia. The protocols were performed in a manner similar to previous reports (19,22,23).

Immunohistochemistry

To histologically confirm the neuroprotective roles of hypothermia, immunohistochemistry was performed in accordance with routine methods, as reported previously (19,24). Briefly, deeply anesthetized rats were perfused transcardially with 4% paraformaldehyde and 0.2% picric acid in a 0.1 M sodium phosphate buffer (PB; pH 6.9). In preliminary experiments, 2 rats for each survival time period, namely 2, 4, and 8 h following ischemia-reperfusion under normothermic or hypothermic conditions, were euthanatized and examined. Based on the preliminary data, we analyzed in detail those that survived for 8 h
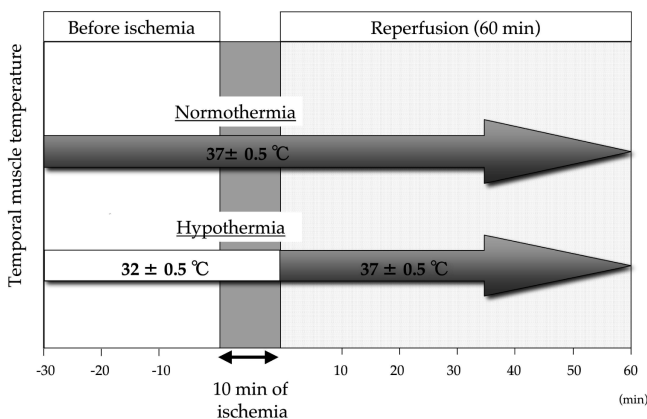
following normothermic ($n = 4$) or hypothermic ($n = 5$) ischemia-reperfusion. The brains were dissected out and immersed in 20% sucrose solution, then transverse sections (20 μm thick) at the hippocampus level were chosen as the target area and cut using a sliding microtome equipped with a freezing stage. The sections were washed overnight in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS), and incubated with rabbit anti-active caspase-3 antibody (Abcam, Cambridge, MA, USA) diluted to 20 μg/ml in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT) for 2 days at 4°C. After washing in PBS, the sections were incubated with a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burligame, CA, USA) diluted 1:100 in PBS-BSAT for 1 h at room temperature. The sections were then washed again in PBS and incubated with avidin-biotin-horseradish peroxidase complex (ABC; Vector Laboratories) diluted 1:200 in PBS-BSAT for 30 min at room temperature. After a final wash in PBS, the sections were reacted with 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer solution (pH 7.4). After counterstaining with thionine, the sections were coverslipped with Malinol (Muto Pure Chemical, Tokyo, Japan). In addition, we analyzed active caspase-3-immunoreactive areas in the nuclei of hippocampal neurons in the CA1 sector using an image-analysis system (Biozero BZ 8000 series, Keyence Co., Osaka, Japan) with fixed square (500 μm^2) sections from each animal.

Statistical analysis

Data are presented as the mean ± S.E.M. Statistical analysis was performed using ANOVA with Fischer's least significant difference test. Differences were considered significant at $P < 0.05$.

Results

Under normothermia, CBF increased to $238.0 \pm 11.6\%$ (Table 1) of the basal level at 13.5 min and gradually decreased thereafter, although it remained at nearly 150% of the basal level for up to 60 min after reperfusion (Fig. 2, top). In contrast, at 6.5 min after reperfusion under hypothermic conditions, CBF increased to $278.0 \pm 29.7\%$, which tended to be earlier and greater than under normothermic conditions, then returned to the basal level within 30 min after reperfusion, which was also different from normothermia (Fig. 2, top). In contrast, the maximum %CBF value after reperfusion did not differ significantly between normothermia and hypothermia (Table 1). In the hypothermia group, temporal muscle temperature was restored to $37.0 \pm 0.5°C$ at around 7 min after reperfusion (Fig. 2, bottom), after which CBF recovered to the basal level within 30 min (Fig. 2, top). As for changes in MABP, there were no significant differences between normothermia and hypothermia throughout the experimental period (Fig. 2, middle).

Pretreatment with MK-801 clearly shortened reactive hyperemia within 25 min, even under normothermic conditions (Fig. 3, top). However, the time taken to reach the maximum %CBF value after reperfusion did not differ significantly between animals under normothermia and those pretreated with MK-801 (Table 1). There were also no significant differences in the changes in MABP during the experimental period between rats pretreated with MK-801 and rats pretreated with saline (Fig. 3, middle).

Active caspase-3-like immunoreactivity in neuronal nuclei in the hippocampal CA1 sector was not remarkable after ischemia-reperfusion in the sham group, and in rats that survived hypothermia for 8 h (Fig. 4A, B). However, marked active caspase-3-like immunoreactivity was observed in the neuronal nuclei of normothermic rats (Fig.

Table 1 The maximum value of % CBF after post-ischemic reperfusion under each condition

	Maximum CBF (%)
Normothermia ($n = 7$)	238.0 ± 11.6
Hypothermia ($n = 7$)	278.0 ± 29.7
Vehicle (saline, $n = 7$)	238.6 ± 20.8
MK-801 ($n = 7$)	232.1 ± 24.3

CBF: Cerebral bloods flow. All values are presented as the mean ± S.E.M. Statistical comparisons were made using ANOVA and Fischer's least-significant-difference test. There were no statistical differences of %CBF between normothermia vs. hypothermia, and vehicle vs. MK-801.
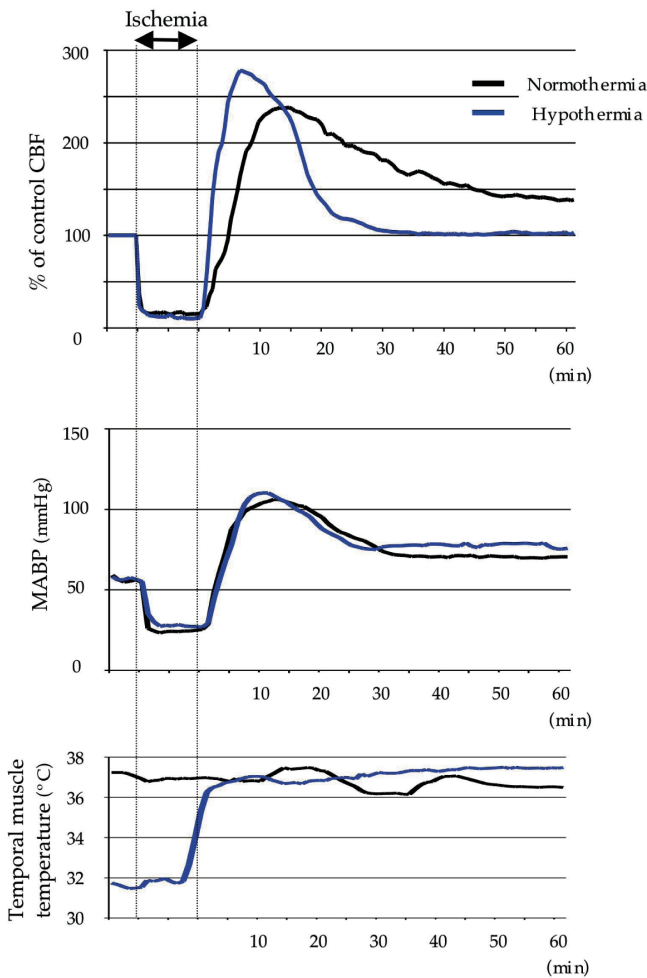
Fig. 2 Changes in %CBF monitored *in vivo* with a laser-Doppler flowmeter (top) and MABP (middle) before, during, and after ischemia-reperfusion in rats under normothermia (black line, $n = 7$) and hypothermia (blue line, $n = 7$). All values are presented as mean values obtained at 1 min intervals. Also shown are representative profiles of changes in temporal muscle temperature (bottom) before, during, and after ischemia-reperfusion in rats under normothermia (black line) and hypothermia (blue line).
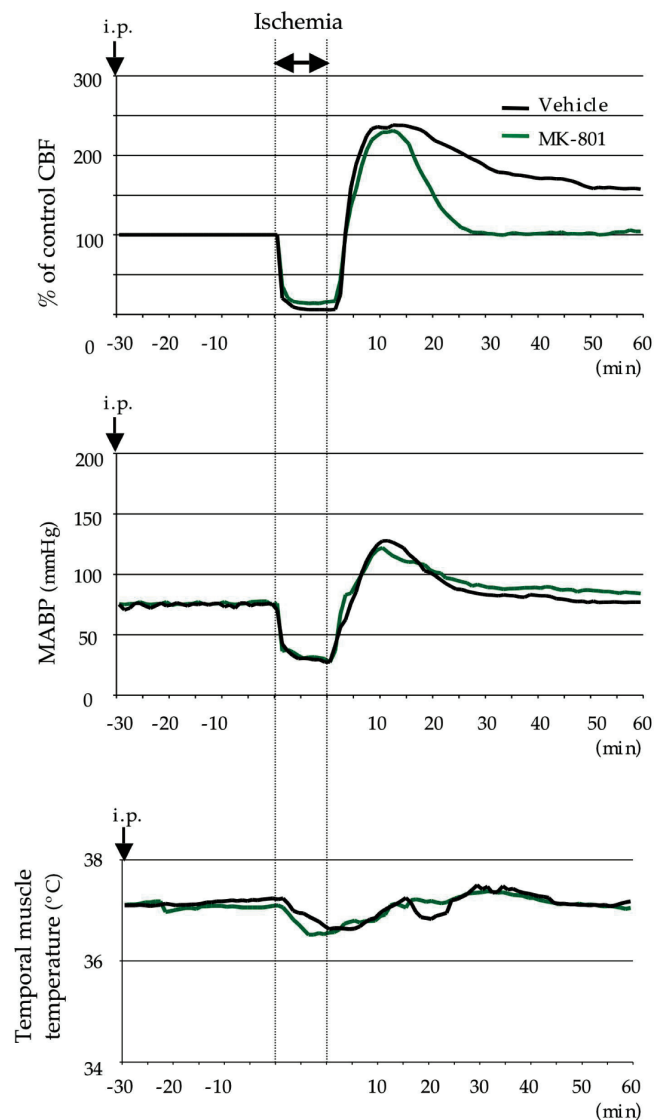

Fig. 3 Changes in %CBF monitored *in vivo* with a laser-Doppler flowmeter (top) and MABP (middle) before, during, and after ischemia-reperfusion in rats pretreated with MK-801 (green line, $n = 7$) or the saline vehicle (black line, $n = 6$) under normothermia. All values are presented as the mean, after averaging the values obtained at 1 min intervals. Also shown are representative profiles of changes in temporal muscle temperature (bottom) before, during, and after ischemia-reperfusion in rats pretreated with MK-801 (green line) or the saline vehicle (black line) under normothermia. Arrows indicate the timing of the intraperitoneal injections of MK-801 (3 mg/kg, $n = 7$) at 30 min prior to ischemia.

4C). In addition, areas in which neuronal nuclei showed active caspase-3-like immunoreactivity in the hippocampal CA1 sector were increased after 8 h of ischemia-reperfusion in the normothermia group, but not in the hypothermia group, as compared with the sham group (Fig. 5).

Discussion

It is well known that reactive hyperemia continues for a few hours after reperfusion (5,6), and a recent study using a hydrogen clearance method to evaluate average hourly changes in cerebral hemodynamics found that moderate cerebral hypothermia (33°C) gradually reduced the degree of reactive hyperemia (25). However, minute changes in reactive hyperemia and the effects of hypothermia during
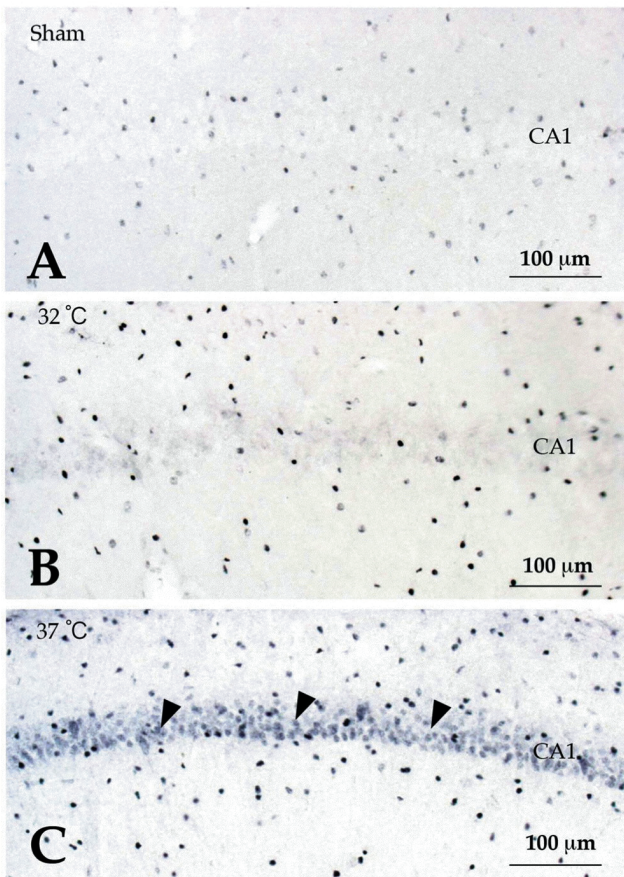
Fig. 4 Representative photograph showing active caspase-3-like immunoreactive neuronal nuclei in the hippocampal CA1 sector of sham-treated (A), hypothermic, (B) and normothermic (C) rats that survived for 8 h after ischemia-reperfusion. Arrows in (C) indicate neuronal nuclei with active caspase-3-like immunoreactivity. Note the presence of neuronal nuclei with caspase-3-like immunoreactivity in the pyramidal cell layer in normothermic ischemia (C), but not in hypothermic ischemia (B), or in the hippocampal CA1 sector of sham-treated rats (A). Scale bars = 100 μm.

Fig. 5 Areas of neuronal nuclei with active caspase-3-like immunoreactivity in the hippocampal CA1 sector of sham-treated (n = 4), normothermic (n = 4), and hypothermic (n = 5) rats that survived for 8 h after ischemia-reperfusion. The results are expressed as the mean ± S.E.M. *Significance vs. sham (P< 0.05). NS: no significant difference.

the initial stage of the post-ischemia reperfusion period (within 60 min) have not been elucidated. Using real-time monitoring of CBF dynamics with a laser-Doppler flowmeter, the present results clearly showed that the peak value of CBF during reactive hyperemia appeared within 15 min under both normothermic and hypothermic conditions. However, the major difference between normothermia and hypothermia was a tendency for the peak value of CBF to be earlier (within 8 min) and greater (up to 270% of the basal level) in hypothermia, perhaps indicating recovery of CBF to the basal level.

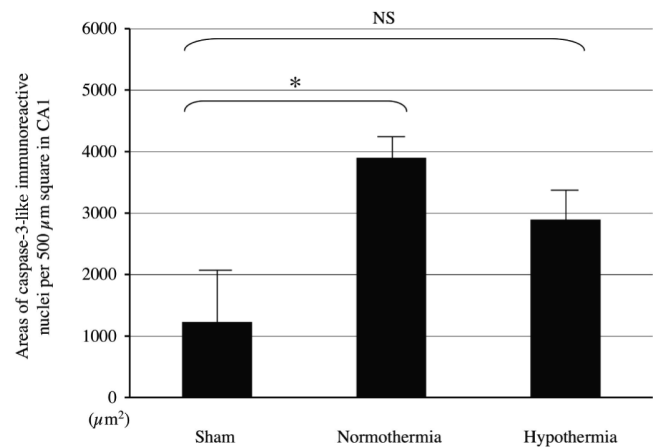[glu]e stimulates post-synaptic neurons and glia, and powerfully activates the synthesis of various vasoactive mediators via calcium-mediated enzymatic activation, which are normal physiological reactions (6). Glutamate itself is not vasoactive, but vasoactive mediators such as PGs and/or NO are synthesized directly by activation of NMDA receptors, and cause smooth muscle relaxation and an increase of local blood flow in the brain (6,17,26-28). We have previously reported that intra-ischemic hypothermia markedly enhanced post-ischemia glutamate re-uptake, resulting in a decrease in the [glu]e content (22). The present results indicate that pretreatment with MK-801 suppresses reactive hyperemia in normothermia, resultng in recovery of CBF to the basal level in a manner similar to hypothermia. Furthermore, MK-801 pretreatment was not associated with an earlier peak or a greater peak value of CBF as compared to the values observed in hypothermia. These results suggest that factors other than the NMDA receptor play a role in the characteristic CBF peak pattern under hypothermic conditions. Regulation of CBF under physiological conditions is very complex, and involves the interaction of multiple factors. In particular, some vasoactive mediators are synthesized from the cascade caused by activation of NMDA receptors, and these regulate CBF, although in other studies blocking of one of these mediators did not completely suppress reactive hyperemia (28-31). Additional investigations will be needed to clarify the factors responsible for the specific CBF profiles induced by hypothermia.

The neuroprotective effects of hypothermia have been demonstrated in a number of studies when the treatment was initiated at the very early stage following ischemic injury (12). Morphological analysis under conditions of global cerebral ischemia similar to those in the present study showed that intra-ischemic hypothermia provided significant neuronal protection, whereas hypothermia induced immediately after reperfusion was less protective (32). Additionally, our immunohistochemical study indicated that hypothermia suppressed the emergence of active caspase-3, which is a major marker of apoptotic neuronal death (14,15) in hippocampal neurons. However, normothermia did not have such an effect in rats that survived for 8 h after ischemia-reperfusion.

In summary, our results indicate that hypothermia shortens the period of reactive hyperemia in the initial ischemia-reperfusion stage, which may be partly associated with activation of NMDA receptors. This phenomenon may provide a beneficial effect that confers resistance against progress of the neurotoxic cascade during the first 8 h after ischemia-reperfusion.

Acknowledgments

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