

Selective Brain Hypothermia Protects against Hypoxic-Ischemic Injury in Newborn Rats by Reducing Hydroxyl Radical Production

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We hypothesized that selective brain hypothermia (SBHT) decreases production of hydroxyl radicals ($\cdot\text{OH}$) induced by hypoxia-ischemia (H-I) and reperfusion and attenuates neuronal damage in neonatal rat brain. Anesthetized 7-day-old rats were divided into a normothermia (NT) group (n=6) and a SBHT group (n=7) and subjected to 90-min H-I, followed by a 90-min recovery period. Brain temperature (BT) was regulated by a water-cooled metallic plate placed under the head. The BT of the SBHT group was set at 31.0 ± 1.0 °C during the H-I and recovery period. Microdialysis and the salicylate-trapping method were used to detect $\cdot\text{OH}$ in the striatum. Neuronal damage was quantified by counting the surviving neurons at 120 hr after reperfusion. The NT group had significant increases in 2,3-dihydroxybenzoic acid (DHBA) ($223\pm 166\%$) and 2,5-DHBA ($321\pm 153\%$) above baseline levels. The increases in 2,3-DHBA ($127\pm 40\%$) and 2,5-DHBA ($133\pm 33\%$) were significantly lower ($p < 0.01$) in the SBHT group. The number of surviving neurons was decreased significantly in the NT group but not in the SBHT group. We conclude that SBHT reduces $\cdot\text{OH}$ production during H-I and reperfusion and has protective effects against neuronal damage.

Brain injury following transient cerebral hypoxia-ischemia (H-I) is mediated by many mechanisms. Treatments for protection against neuronal cell death induced by H-I and reperfusion have been developed in recent years, but none has been highly successful. A fundamental process believed to be responsible for H-I damage to neurons is excitotoxicity, triggered mainly by elevated extracellular glutamate concentration (7). Recent evidence suggests that immature white matter can be damaged by excitotoxicity (11, 14). Reactive oxygen species are generated within brain tissue during H-I and play a role in the development of cerebral damage. They may be directly involved in glutamate release (31) and, more importantly, they may participate in the excitotoxic process itself. These oxygen species are extremely reactive and attack lipids, proteins, and nucleic acids, which results eventually in tissue injury and cell death. Myelin, oligodendrocytes, and axons are main constituents of white matter. Myelin, which is rich in lipids, is susceptible to lipid peroxidation. Hydroxyl radical ($\cdot\text{OH}$) is the most reactive oxygen metabolite in cerebrum and plays a key role in the development of cerebral damage.

Hypothermia applied during or after H-I is thought to protect against neuronal damage. The effect of hypothermia is thought to relate in part to a reduction in the release of excitotoxins and production of oxygen free radicals (19, 20, 22). Recent studies have shown

that hypothermia also suppresses caspase-3 activity (12) and inhibits H-I-induced apoptosis (9, 29). Hypothermia may attenuate the excitotoxic cascade by altering processes favoring apoptosis (2, 8, 34), or it may work in adults animals through other mechanisms such as reduction of glutamate release and $\cdot\text{OH}$ production (22).

Most hypothermia studies in neonatal animals have examined the effect of whole-body cooling. They leave unanswered whether selective head cooling is an effective treatment in developing brain. The present study tests the hypothesis that selective brain cooling decreases production of hydroxyl radicals induced by H-I and reperfusion and attenuates neuronal cell death in neonatal rat brain. We determined the production of $\cdot\text{OH}$ during H-I and reperfusion using a microdialysis method.

METHODS

Animal Model

We used 7-day-old Sprague-Dawley rats (Charles River Japan, Yokohama, Japan). After the rats were anesthetized with urethane (Tokyo Kasei, Tokyo, Japan, 1.125 g/kg, i.p.), a sinistral neck incision was made, and the left common carotid artery was exposed, and loosely encircled with a 3-0 surgical nylon suture. After an incision was made along the midline of the scalp, the scalp was reflected to expose the skull, and a 1.5-mm diameter hole was drilled into the skull over the partial hemisphere. Following placement of the animal's head in a stereotaxic frame (Narishige Instruments, Tokyo, Japan), a microprobe (PF319, Perimed, Stockholm, Sweden) for the measurement of regional cerebral blood flow (rCBF), a temperature probe (OD 0.6 mm; Unique Medical, Tokyo, Japan) and a microdialysis probe (OD 0.24 mm; CUP11, CMA/Microdialysis AB, Sweden) were implanted into the left striatum with a micromanipulator (Narishige Instruments, Tokyo, Japan). The stereotaxic coordinates were as described by Andersen and Gazzara (1): (A, 1.0 mm from the bregma; L, 2.7 mm from the bregma; and D, 4.5mm from the dura). Another temperature probe was placed in the rectum. All experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine and were approved by the local committee on animal experimentation.

Experimental Design

Transient Hypoxia/Ischemia and Reperfusion

After surgery, a 2-h period was allowed for stabilization. H-I was produced by occlusion of the left common carotid artery with an aneurysm clip (Sugita miniclip, Mizuho, Tokyo, Japan) and by exposure to 8% O₂. The concentration of inhaled O₂ was monitored continuously with an oxygen monitor (OX-21, ATOM medical, Tokyo, Japan). Reperfusion and reoxygenation were achieved by unclamping the artery and exposing the animal to room air.

Selective Brain Hypothermia

The rats were divided into a normothermia (NT) group (n=6) and a selective brain hypothermia (SBHT) group (n=7). The rat's head was secured to a metallic plate brain thermo-regulator (BTC-201S, Unique Medical Co., Tokyo, Japan). Brain temperature was monitored continuously by the thermo-regulator throughout the experiment. Head cooling in the SBHT group was started at the beginning of H-I by cooling the metallic plate, and brain temperature was maintained at 30 to 32°C until 90 min after the start of reperfusion. Rectal temperature was maintained at 35 to 36°C throughout the experiment with a thermostatically controlled heating blanket (ATC-101B, Unique Medical) and a heat lamp. In the NT group, brain temperature was maintained at 35.5 to 36.5°C.

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Measurement of rCBF

During the experimental period, rCBF in striatum was measured by laser Doppler flowmetry with a PeriFlux 4001 Master flowmeter (Perimed, Stockholm, Sweden). Relative flow values were expressed as perfusion units. All time course data are expressed as percentages of baseline levels.

Measurement of $\cdot\text{OH}$

We used the salicylate-trapping method to detect extracellular $\cdot\text{OH}$ by measurement of the stable adducts 2,3- and 2,5-dihydroxybenzoic acid (DHBA) in the dialysates (10, 30). The microdialysis probe in striatum was perfused with Ringer's solution (pH 7.4; 147 mM NaCl, 4 mM KCL, 2.3 mM CaCl₂) containing 0.5 mM sodium salicylate (Sigma Chemical Co., St. Louis, MO) at a flow rate of 2 $\mu\text{l}/\text{min}$. Dialysate samples were collected at 15-min intervals, and assayed for 2,3- and 2,5-DHBA by HPLC with electrochemical detection.

Neuronal Cell Counts

In the H-E sections of brains fixed 120 hr after reperfusion, neuronal damage was quantified by counting the surviving neurons in both groups at the cerebral cortex, CA1 pyramidal region in the hippocampus (A, -2.0 mm from the bregma), and striatum (A, 1.0 mm from the bregma) levels. Briefly, only neurons with a well-defined nucleus and an intact cell membrane of the ipsilateral brain were counted. Sections were viewed with a light microscope (400x) connected to a personal computer. Randomly selected fields were digitized with a digital camera (Fujix HC-300Z, Fuji Film, Tokyo, Japan) and displayed on a monitor by means of commercial software (Photograb-300Z Fuji Film). Cell counting was performed by marking cells displayed on the monitor. If any part of a cell touched the edge of the field of the view, it was not counted. Finally, total number of cells was summed across two separate fields (total area 20,000 μm^2). The data are presented as the mean neuronal cell count per field.

Statistical Evaluation

Values of are expressed as mean and standard deviation. Statistical analysis was done with Stat-View version 5.0 for the Macintosh (SAS Institute Inc. Carey, NC). Difference between time periods were analyzed by ANOVA with repeated measures followed by Fisher's protected least significant difference test. Multiple comparisons between groups were done with Bonferroni correction. In all analyses, $p < 0.05$ was considered statistically significant.

RESULTS

Brain and Rectal Temperatures

Head cooling was started at the initiation of hypoxia and ischemia. It took 1 hr to keep brain temperature 30~32°C. But, after that, brain temperature was able to be kept 30~32°C during 90-min recovery period. Rectal temperature in the SBHT group was decreased (by 1.7°C on average) significantly (Figure 1).

Effect of SBHT on rCBF

The rCBF values during H-I and the recovery period are shown in Figure 2. In both groups, rCBF decreased significantly during H-I and increased to the baseline level during reperfusion. At the beginning of reperfusion, rCBF was higher than baseline only in the NT group. The rCBF tended to be lower in the SBHT group than in the NT group throughout the experiment. However, the difference between the two groups was not significant.

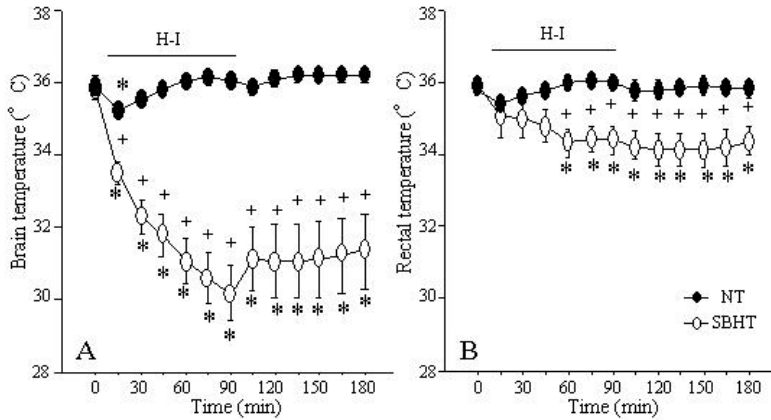


Figure 1

Changes in brain and rectal temperature due to head cooling, which was started at the initiation of hypoxia and ischemia. Brain temperature decreased to 30-32°C (A). Rectal temperature decreased slightly during head cooling (average -1.7°C) (B). * $p < 0.05$ vs. baseline, + $p < 0.05$ the normothermia (NT) group vs. the selective brain hypothermia (SBHT) group. Values are mean \pm SD.

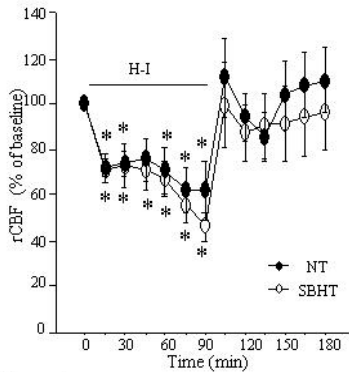


Figure 2

Changes in regional cerebral blood flow in striatum. Hypoxia-ischemia was performed for 90 min. rCBF decreased during hypoxia-ischemia and increased to the baseline level during reperfusion. In the selective brain hypothermia group, rCBF was never above the baseline level after reperfusion. * $p < 0.05$ vs. baseline. Values are mean \pm SD.

Effect of SBHT on Hydroxyl Radical Production

The NT group had significant increases in the levels of 2,3-DHBA ($223 \pm 166\%$) and 2,5-DHBA ($321 \pm 153\%$) relative to and of baseline levels during H-I and reperfusion. The levels reached a maximum 60 min after the initiation of H-I (Figure 3). In the SBHT group, 2,3- and 2,5-DHBA did not increase significantly during H-I and reperfusion. The peaks in 2,3-DHBA ($127 \pm 40\%$) and 2,5-DHBA ($133 \pm 33\%$) were significantly lower and came later than those in the NT group ($p < 0.01$ vs. the NT group).

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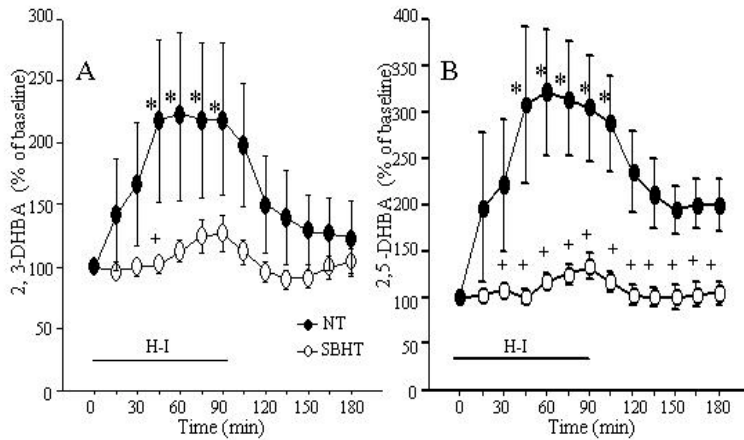


Figure 3

Changes in $\cdot\text{OH}$ in striatum due to hypoxia-ischemia. In the normothermia group, 2,3- (A) and 2,5- (B) DHBA increased as soon as hypoxia-ischemia insult began, not only after reperfusion. The DHBA's did not return to baseline levels during reperfusion. * $p < 0.05$ vs. baseline, + $p < 0.05$ the normothermia vs. the selective brain hypothermia. Values are mean \pm SD.

Neuronal Damage

The mean numbers of surviving neurons per field in cortex, hippocampus, and striatum 120 hr after reperfusion are shown in Figure 4. In the NT group, a distinct decrease in cell numbers in cortex, hippocampus, and striatum was found compared with a sham control (11.4%, 9.0% and 21.8% respectively, $p < 0.0001$) and with the SBHT group (11.9%, 9.9% and 22.7% respectively, $p < 0.0001$). In the SBHT group, the numbers of surviving neurons were not decreased significantly compared to those in sham operated animals.

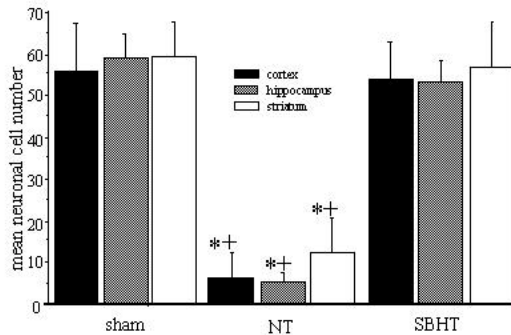


Figure 4

Surviving neurons in the ipsilateral region 120 hr after reperfusion. In the normothermia group, the number of surviving neurons decreased significantly in all three regions. Between the sham group and the selective brain hypothermia group, no significant difference in the number of surviving neurons was seen. * $p < 0.0001$ vs. sham, + $p < 0.0001$ vs. the selective brain hypothermia. Values are mean \pm SD.

DISCUSSION

In the present study, SBHT resulted in protective effects against neuronal damage after H-I and reperfusion by reducing $\cdot\text{OH}$ production. We assessed neuronal damage by counting surviving neurons 120 hr after reperfusion. Sufficient protective effect was detected histologically in the SBHT group.

The neuroprotective effect of hypothermia has been recognized for a long time. Since Busto et al. reported in 1987 that hypothermia of 33°C has a neuroprotective effect (4), much attention has been paid to moderate hypothermia for the treatment of H-I brain injury (2, 26, 27). Not only whole body hypothermia but also SBHT has neuroprotective effects in adult animals (17, 28, 36). Several recent studies have shown the effect of hypothermia in immature animals (32, 33). However, the effects of SBHT on the reduction of $\cdot\text{OH}$ production and neuronal damage have not been observed in immature animal brain.

Therapeutic intervention with hypothermia after H-I injury in the immature animal brain is not an established procedure. In the present study, because it took 1 hr to keep brain temperature 30~32°C, we initiated head cooling at the beginning of H-I. During the hypothermic experiment, rectal temperature decreased significantly compared to that of the NT group, although a heat blanket was used to keep the body warm. This temperature drop was probably due to suppression of the homeostatic metabolic response to head cooling (3).

Some investigators have concluded that the neuroprotective effect of hypothermia is due to the decrease in rCBF (18, 35). In our study, rCBF tended to be lower in the SBHT group than the NT group, but the difference was not significant. Such a decrease may be due partly to the lowered metabolic rate and reduced energy depletion caused by head cooling. Kil et al. showed that the effect of brain temperature on $\cdot\text{OH}$ production did not correlate with the alternation in rCBF (22). Brain temperature-related attenuation of $\cdot\text{OH}$ production may contribute to the neuroprotection afforded by SBHT.

It is widely understood that production of free radicals is increased by H-I and reperfusion (15). It has been shown that hydroxylation of salicylate by $\cdot\text{OH}$ leads to the formation of both 2,3- and 2,5-DHBA, and the concentrations of these adducts increase during H-I and reperfusion in cortex, hippocampus and striatum (6, 13, 37). The microdialysis technique we used to detect $\cdot\text{OH}$ in brain (10, 16) enabled us to measure $\cdot\text{OH}$ production continuously during H-I and the recovery period. $\cdot\text{OH}$ production during H-I and reperfusion increased significantly above baseline in the NT group but not in the SBHT group. The conjecture that activation of glutamate receptors and glutamate excitotoxicity is associated with free radical formation is supported by several recent studies (24, 25). Direct activation of N-methyl-D-aspartate (NMDA) receptors results in a massive release of $\cdot\text{OH}$. Normothermic ischemia leads to a massive surge in extracellular glutamate that is suppressed by hypothermia (5). The elevation in extracellular glutamate occurs primarily during the intraischemic period. In our study, $\cdot\text{OH}$ production also increased primarily during the intraischemic period. The peak increased $\cdot\text{OH}$ production came 30 min sooner in the NT group than that in the SBHT group. This result is consistent with previous studies that have shown that moderate hypothermia reduces the initial rate of rise in extracellular K^+ concentration, delays terminal depolarization (21), and delays cellular Ca^{++} uptake (23). It is not certain how hypothermia reduced $\cdot\text{OH}$ production in this experiment. Further studies are needed to determine which mechanism is primarily responsible for the production of $\cdot\text{OH}$ in rat brain during H-I and reperfusion.

Our experiments were not designed to observe adverse hypothermic effects. In adult animals, it has been shown that there are no significant alterations in physiological parameters such as systemic blood pressure, arterial blood gases, hematocrit, and blood

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glucose level during selective head cooling (18). Nevertheless, given the multitude of factors that affect neuroprotection, it is evident that the optimal hypothermic parameters for the best outcome with minimal adverse effects will have to be worked out.

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