Combination of Intraischemic and Postischemic Hypothermia Provides Potent and Persistent Neuroprotection Against Temporary Focal Ischemia in Rats

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Background and Purpose—It is not known whether a combination of intraischemic and postischemic mild hypothermia provides extra neuroprotection and if so, whether the neuroprotection is persistent.

Methods—Sixty-eight Sprague-Dawley rats were used. In group 1, ischemia and reperfusion were performed under normothermic (N) conditions (control, N-N). In group 2, ischemia was induced and maintained under hypothermic conditions (33°C for 2 hours) and reperfusion was performed under normothermic conditions, H-N. In group 3, both ischemia and reperfusion were performed under hypothermic conditions for an additional 21 hours after the surgery, H-22H. In group 4, ischemia was induced and maintained under hypothermic conditions and reperfusion was performed under hypothermic conditions only for the initial 3 hours (H-3H). In group 5, ischemia was induced and maintained under normothermic conditions and reperfusion was performed under hypothermic conditions (33°C) (N-22H). All rats were perfused 48 hours after the induction of ischemia. In addition, the normothermic or hypothermic therapy used for groups 1, 3, and 4 was performed again, and these rats were killed 30 days after the induction of ischemia. Furthermore, neurological deficits were monitored in groups N-N and H-22H for 4 weeks.

Results—In the H-3H and H-22H groups, the total infarct volume was significantly reduced by 41% or 66%, respectively, assessed 48 hours after ischemia. The significant reduction in group H-22H was again confirmed 30 days after ischemia, ie, 50% reduction was observed. In contrast, the reduction in group H-3H (31%) was not significant. The neurological deficits were significantly more severe in the N-N group than in the H-22H group during week 4.

Conclusions—The neuroprotective effects against temporary focal ischemia evaluated by infarct volume and neurological functions by the combination therapy with intraischemic and prolonged postischemic mild hypothermia were persistent in rats. Appropriate design of mild hypothermia therapy extending into the late reperfusion period is important to maximize the neuroprotective effects of hypothermia. (Stroke. 1999;30:2720-2726.)

Key Words: hypothermia ■ cerebral infarction ■ cerebral ischemia ■ rats

A treatment using hypothermic conditions has been recognized to be quite potent in protecting neurons against lethal ischemic stress. Intraischemic hypothermia has been shown to be neuroprotective in global1–3 and focal cerebral ischemia.4–6 Even when hypothermia is initiated only after temporary cerebral ischemia (postischemic hypothermia), a significant neuroprotective effect has been observed in both global and focal cerebral ischemia.7–9 In postischemic hypothermia, a prolonged application of hypothermia seems to be necessary to achieve significant and persistent neuroprotection for global ischemia. Colbourne and Corbett10 reported that ≈90% hippocampal CA1 neuroprotection was achieved by 1 hour–delayed mild (32°C) postischemic hypothermia for 24 hours, compared with only ≈15% neuroprotection by 1 hour–delayed postischemic hypothermia for 12 hours. The CA1 neuronal death was assessed 30 days after global ischemia in gerbils. In temporary focal ischemia, we previously demonstrated a significant (32%) reduction of the volume of cerebral infarct on immediate and mild (32°C to 33°C) postischemic hypothermia for 21 hours. In contrast, no reduction was observed after immediate postischemic mild hypothermia for only 1 hour, and only a small reduction (22%, P=NS) on 30 minutes–delayed postischemic mild hypothermia for 21 hours assessed 48 hours after the induction of temporary focal ischemia in Sprague-Dawley rats.7 The use of either intraischemic or prolonged postischemic hypothermia alone protects neurons from ischemic injury; however, it is unknown whether a combination of intraischemic and postischemic hypothermia provides extra neuroprotection and if so, whether the neuroprotection is persistent.

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mic and postischemic hypothermia provides further neuroprotection, and if so, whether the effects persist. Because of the technical complexity of establishing a reliable model of focal ischemia combined with prolonged temperature regulation under general anesthesia, few studies have been performed to clarify the efficacy of such combined hypothermia therapy. The present study was conducted to elucidate the effects of brief or prolonged mild postischemic hypothermia in combination with intraischemic mild hypothermia on neocortical infarct size or neurological deficits in a temporary focal ischemia model in rats. 11

Materials and Methods

Experimental Groups

A total of 68 male Sprague-Dawley rats (SLC, Kyoto, Japan) weighing 300 to 350 g were used. Fifty-six rats were assigned randomly to 8 groups of 7 rats each. Temporary focal ischemia was induced with a microclip on the left middle cerebral artery (MCA) in combination with temporary occlusion of the bilateral common carotid arteries (CCAs) for 2 hours. In group 1, ischemia and reperfusion were maintained under normothermic (N) conditions (control, N-N). In group 2, ischemia was induced and maintained under hypothermic (H) conditions (32°C to 33°C), and reperfusion was performed under normothermic conditions (H-N). In group 3, both ischemia and reperfusion were performed under hypothermic conditions (32°C to 33°C for 2+1 hours during surgery, and then rats were housed in a cold room, 6°C, for an additional 21 hours after the surgery, H-2H). The induction of hypothermia was always achieved by alcohol application to the body during anesthesia. Housing in a cold room thereafter, the low-temperature environment, significantly prevented the recovery of body temperature and maintained hypothermia under awake conditions. The temperature of the cold room was set at 12°C from 22 hours to 46 hours after ischemia to avoid the rebound phenomenon in the prolonged postischemic hypothermia group. 7 In group 4, ischemia and the initial reperfusion were performed under hypothermic conditions (32°C to 33°C for 3 hours postischemia), and then rats were housed in a normothermic room (25°C) (H-3H). In group 5, ischemia was induced and maintained under normothermic conditions, and reperfusion was performed under hypothermic conditions for 22 hours (N-2H). All of these rats were killed 48 hours after the induction of ischemia. In an additional study, the persistence of the effect of the combined hypothermic therapy was assessed. The same normothermic or hypothermic experiments done with groups 1, 3, and 4 were performed in groups 7, 8, and 9, and these rats were killed and the volume of cerebral infarct was analyzed 30 days after the induction of ischemia.

In an additional experiment, another 12 rats were divided into 2 groups (n=6 each), and cerebral functions (neurological deficits) after ischemia in normothermic or combined and prolonged mild hypothermic groups were analyzed as described below.

Surgical Protocols

All the rats had access to food and water ad libitum until surgery. The surgical procedures used to induce temporary focal neocortical ischemia were described in detail elsewhere. 11 Briefly, anesthesia was induced with halothane in a mixture of oxygen and nitrogen. The right femoral artery was cannulated in every rat to monitor mean arterial blood pressure (model AP-611G, AP-600G, Nihon Kohden), assay blood gases (PO2, PCO2, pH; model ABL300, Radiometer Copenhagen), and measure the concentration of blood sugar. The mean arterial blood pressure was kept within 100 to 120 mm Hg by adjustment of the halothane concentration. A digital thermometer was used to monitor rectal temperature beginning just before MCA clipping; the temporal muscle temperature was simultaneously monitored with a digital thermometer. Both temperatures were controlled within 36.5°C to 37.5°C in the normothermic condition and within 32.0°C to 33.0°C in the hypothermic condition with a heating lamp or alcohol application to the body surface during surgery. With a surgical microscope, both CCAs were exposed, and a snare was placed loosely around each CCA with 5-0 nylon thread (PES0, Nippon Becton Dickinson). After the CCA snare had been prepared and mechanical ventilation set up, the left MCA was exposed by craniectomy and clipped with an arterial clip (Sundt AVM Microclip No. 1, Codman) at the lateral border of the olfactory tract. Immediately after the clipping of the MCA, the CCA snares were pulled to occlude the CCAs. After 2 hours of 3-vessel occlusion, the microclip was removed and the snares were released. Reflow of the left MCA and both CCAs was confirmed visually during surgery.

The experimental protocols were approved by the animal research committee at the National Cardiovascular Center Research Institute. All efforts were made to minimize suffering and the number of animals used.

Analyses of Infarct Volume

All animals were administered an overdose of sodium pentobarbital and perfused intracardially with 200 mL of ice-cold heparinized 10 mmol/L sodium phosphate–buffered saline (pH 7.5) (PBS) at ~110 to 140 mm Hg. The brain was removed, cut from the frontal tip into slices 2 mm thick (RBW-4000C, ASI Inst), and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC). The stained slices were then fixed by immersion in phosphate-buffered 4% paraformaldehyde/PBS. When the cerebral infarct was assessed 48 hours after ischemia, the infarct area and hemispheric areas of each section were traced under a stereomicroscope and measured with an image-analysis system (SD-510C, Wacom). An edema index was calculated by dividing the total volume of the hemisphere ipsilateral to the MCA occlusion by the total volume of the contralateral hemisphere. An infarct index, ie, the actual infarct volume adjusted for edema, was calculated in each animal as the total infarction volume divided by the edema index. When the assessment of cerebral infarction was done at the chronic stage, 30 days after ischemia, the surviving neocortical area was assessed by cresyl violet stain or TTC. The volume of the right normal neocortex was measured and subtracted from the left neocortical volume to calculate the total infarct volume, because the infarct-necrosis area was completely liquefied and absorbed. Before the procedure of paraffin-embedding of the brain slices, the shrinkage (rate) of the brain by dehydration was measured in the right hemisphere to calculate the absolute infarct volume. In addition, the gliosis that developed within 30 days after the ischemic injury was visualized with glial fibrillary acidic protein (GFAP) staining in the same brain sections. In the measurement of the intact left neocortex by cresyl violet at the chronic stage, the area of gliosis (developed in the missing area) was excluded from the area of intact neocortex.

Analyses of Cerebral Functions

Neurological deficits were examined according to the scoring scale described by Yamamoto et al, 12,13 with modification, 2 days and 1, 2, 3, or 4 weeks after ischemia in the normothermic (N-N) group and in the intra- and prolonged (22 hours) posts ischemic hypothermia (H-2H) group. Hemiplegia and posture while being lifted by the tail were graded according to the following criteria: 0, no deficit (symmetrical movement); 1, mild deficit (symmetrical incomplete flexion of the right forelimb or leftward-twisting tendency of the body); and 2, severe deficit (asymmetrical complete flexion of the right forelimb or repeated leftward body twisting). The neurological deficit score was the sum of the hemiplegia and posture grades (from 0 to 4).

Statistics

Physiological data (ie, blood pressure, gases, blood sugar concentration, and pH), regional cerebral blood flow at each time point, infarct volumes, and infarct indexes were analyzed by ANOVA. If multiple comparisons were indicated, the Student-Newman-Keuls test was applied. The neurological deficit score at each time point was analyzed by unpaired t test. The results are presented as mean±SEM. A value of P<0.05 was considered significant.
Results

The physiological parameters, except the temporal muscle and rectal temperatures, were controlled within the physiological range (data not shown). There were no significant differences in such parameters among the groups. The rectal (core) temperatures for 48 hours in all groups are shown in Figure 1. In both postischemic hypothermia groups (N-H and H-H), the core temperatures were maintained at \( \approx 35^\circ C \) to \( 36^\circ C \) from 6 hours after the reperfusion. Spontaneous hyperthermia (\( \approx 38^\circ C \)) was observed in rats housed under normothermic conditions 6 hours beyond the induction of the temporary focal ischemia. Forty-eight hours after the induction of ischemia, the core temperatures were similar in all 4 groups.

Analyses of Cerebral Infarct

In the H-N, H-22H, and N-22H groups, 1 of the 7 rats in each group died before an assessment of cerebral infarct 2 days after ischemia. The autopsy of these 3 rats did not reveal any abnormality capable of causing death. The cortical infarct that could be assessed at the designated time was clearly demarcated as a pale area, in contrast to the surrounding intact red area, when assessed 2 days after ischemia (Figure 3A). In the additional experiment, no rat died before the assessment 30 days after ischemia.

The average infarct volumes for each group are shown in Figure 2. The size of the cerebral infarct was significantly reduced only by a combined therapy of intraischemic and postischemic mild hypothermia. There were significant differences between the control (N-N) and H-22H or H-3H groups; ie, the combination therapy of intraischemic and postischemic hypothermia was effective in reducing the infarct volume. Although the average infarct volume in group H-22H was smaller than that in group H-3H, the difference did not achieve significance. The average infarct volume and the infarct index for each group are shown in the Table. The significant differences observed in the infarct volumes among the groups were also observed among the infarct indexes.

Thirty days after ischemia, the cerebral infarct lesion was noted as a shrunken neocortex or a missing area in the neocortex (Figure 3B). In the border of the lesion after necrosis, a thin layer of glial proliferation was observed surrounding the GFAP-negative tissues (data not shown). The infarct index obtained by cresyl violet stain (corrected by the shrinkage rate: 1.51 on average) is shown in Figure 4. The infarct index obtained by the TTC stain (before dehydration) is shown in Figure 5. The absolute infarct values in each analysis were almost identical, ie, the differences in the values derived by the 2 analyses (cresyl violet and TTC) were 4, 3, or 1 mm\(^3\) on average in the N-N, N-22H, or N-3H groups, respectively. In both analyses of the cerebral infarct, there were significant differences between groups N-N and H-22H but not between groups N-N and H-3H.

Analyses of Cerebral Function

Severe neurological deficits were observed in the acute phase after temporary focal ischemia in the normothermia (N-N) group but were gradually recovered in the observation period (Figure 6). In contrast, in the hypothermic group (H-22H), no or slight neurological deficits were observed from the acute phase after temporary focal ischemia and were consistent throughout the whole observation period. There was a significant difference in the neurological deficit scores at each time point between the groups for 4 weeks after induction of ischemia.

Discussion

In temporary focal ischemia, Xue et al,\(^{14}\) using intraischemic moderate (31°C) hypothermia for 3 hours with subsequent normothermic (37°C) reperfusion for 21 hours, reported profound neuroprotection. The mean infarct volume was reduced to 8% of control (17±12 mm\(^3\) from the control value of 211±35 mm\(^3\)) in a 3-vessel occlusion model in rats. They also tried postischemic moderate (31°C) hypothermia for 3 hours, which resulted in a significant reduction of the infarct volume, 39% (65±54 mm\(^3\)) of control (166±27 mm\(^3\)); however, it was assessed 3 hours after ischemia because of
the high mortality rate under general anesthesia. Since then, it has been thought that intraischemic hypothermia was more effective than postischemic hypothermia in a temporary focal ischemia model. However, the present results showed that postischemic mild hypothermia for 22 hours had a great neuroprotective effect (36% reduction of the control infarct volume) at the level that achieved a significant difference from the control value, and the reduction rate was similar to that after intraischemic mild hypothermia (27% reduction). However, the difference from the control volume was not significant, because larger numbers of experimental groups were used in this study than in the previous study. In the literature, when postischemic hypothermia was delayed or applied for only a brief period, the neuroprotective effects varied. When immediate and moderate (30°C) postischemic hypothermia for 3 hours after focal ischemia was used, the neocortical infarct was significantly reduced when assessed 7 days after ischemia. In contrast, Dietrich et al found that acute (within 3 minutes into the reperfusion) postischemic moderate (30°C) hypothermia for only 3 hours failed to protect CA1 neurons assessed 7 days or 2 months after global ischemia in rats. Furthermore, Welsh and Harris reported that acute (within 10 minutes into the reperfusion) and deep (23°C) postischemic hypothermia for 2 hours failed to protect hippocampal CA1 neurons assessed 7 days after global ischemia in gerbils. In general, prolonged systemic anesthesia is necessary to control the body temperature; however, it leads to deterioration of the general condition of small animals. To overcome this, we used a cold room for a temporary focal ischemia model after the end of anesthesia to keep the body temperature low for a prolonged period during the reperfusion. The core temperature was effectively reduced in the cold room and was 2°C to 4°C lower than that of animals kept in a normothermic room for 24 hours after

<table>
<thead>
<tr>
<th>Group</th>
<th>Infarct volume, mm³</th>
<th>Infarct index, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-N</td>
<td>219±17</td>
<td>204±15</td>
</tr>
<tr>
<td>H-N</td>
<td>159±25</td>
<td>147±22</td>
</tr>
<tr>
<td>H-22H</td>
<td>76±26*</td>
<td>70±23*</td>
</tr>
<tr>
<td>H-3H</td>
<td>127±26*</td>
<td>121±23*</td>
</tr>
<tr>
<td>N-22H</td>
<td>141±23</td>
<td>133±20</td>
</tr>
</tbody>
</table>

All rats were killed 2 days after ischemia. The data are mean±SEM.

Figure 3. A, Brain slices of group N-N (top) and group H-22H (bottom) 2 days after ischemia. The infarcted brain areas (white areas) were visualized by TTC. B, Brain slices of N-N (top) and H-22H (bottom) 30 days after ischemia. Neurons were stained (blue) by cresyl violet, and reactive astrocytes and developed gliosis were visualized by GFAP staining.
surgery. From the present results, it was demonstrated that the acute (3 hours) reperfusion period after focal ischemia was important for the achievement of neuroprotection by mild hypothermic therapy. The reduction rate of 66% observed in the present combination therapy was striking compared with that using only intraischemic moderate (22%) or intraischemic deep hypothermia (50%) therapy.

To judge the neuroprotective effects, delayed assessment (1 to 6 months) of the ischemic neuronal injury has been considered important in global ischemia. Recently, a delayed development of cerebral infarction has been reported after mild focal ischemia. In the present study using temporary focal ischemia, when the effect of neuroprotection was assessed at a later time point (30 days after the onset), the significant reduction in group H-22H, a 66% reduction, observed on day 2 was found to have persisted, ie, 50% reduction was observed 30 days after ischemia. Conversely, 31% reduction was observed 30 days after ischemia in group H-3H, which was not significant compared with that of the normothermic control (group N-N). There was 10% to 16% growth of the neocortical infarct volume in 28 days (compared with the control infarct volume) after the combined mild hypothermia therapy. The results indicated that the significant neuroprotective effect on the infarct volume achieved with the combination of mild intraischemic and prolonged postischemic hypothermia therapy was not transient. Furthermore, functional impairments caused by temporary focal ischemia under normothermic conditions were also prevented by the combined prolonged hypothermic intervention.

It is unknown how hypothermic treatment protects neurons from lethal ischemic stress. When intraischemic hypothermia was applied, no significant difference was reported in high-energy phosphate depletion or in levels of lactate accumulation at the end of the ischemic insult. Several mechanisms regarding hypothermic brain protection have been postulated, including reduced release of glutamate, reduced incidence of spreading depression, improved regional cerebral blood flow during acute reperfusion, early restoration of inhibited protein synthesis, and decreased production of free radicals. Enhanced cerebrovascular permeability is reported after ischemia, and it was reported that intraischemic hypothermia reduced the abnormal enhancement of the blood-brain barrier permeability during and after ischemia. Furthermore, secondary elevation of the extracellular glutamate level or calcium-activated proteolysis in neurons during reperfusion after focal ischemia has been reported. The activation of protein kinase C during focal ischemia was reported, and it was suppressed by an application of moderate hypothermia in rats. We had observed that the activity of protein tyrosine phosphatase was irreversibly reduced in the ischemic core after reperfusion, and to a lesser extent in the penumbral area, and that both of these reductions were prevented by mild hypothermia treatment. The finding...
in this study was in line with those of others in which a prolonged period of hypothermia was important to achieve persistent neuroprotection.\textsuperscript{7,10,18,39} Some key enzymes that are essential for neurons to lead to death may be thermosensitive, i.e., they are suppressed by mild hypothermia, and a prolonged inhibition of unknown (enzymatic) “death cascades” by intraschismic and postischemic hypothermia may cause an alteration of these reactions in different directions that results in neuronal survival. A prolonged inhibition of these reactions may ultimately interrupt the death cascades in neurons triggered by severe ischemic stress.

A gradual rewarming of the body temperature was strictly performed in the present treatment of prolonged hypothermia. A sudden elevation of the surrounding room temperature deteriorated the systemic condition, causing death, as mentioned in a previous report.\textsuperscript{7} Artificial temperature control for a prolonged period should be done with extremely careful observation to avoid any deteriorating complications and to achieve good outcomes.\textsuperscript{40}

It has already been postulated that ischemic cerebral injury is an ongoing process from the induction of global ischemia through the acute reperfusion period.\textsuperscript{7,10,39} In the present study using temporary focal ischemia, mild and prolonged hypothermia therapy prevented neuronal death during ischemia and also prevented neuronal death processing during the reperfusion period. Ischemic brain attack (focal cerebral ischemia) in clinical situations is not always transient; however, induction of reperfusion for the occluded vessel with thrombolytic agents is the main therapeutic modality.\textsuperscript{41–44} In cases in which reperfusion is to be achieved, the application of mild hypothermia therapy initiated in the initial ischemic period has the potential to rescue a large part of the brain from the development of cerebral infarction. However, it is necessary to clarify the effects of prolonged mild hypothermia on permanent (not temporary) focal ischemia to be able to apply this strategy in the ultra-acute phase of stroke before the achievement of active reperfusion, because whether ongoing focal ischemia is transient when hypothermic therapy is initiated is unpredictable. Recently, a beneficial effect of mild prolonged hypothermia initiated at the acute phase after the diagnosis of cerebral infarct on severe permanent cerebral infarction in humans has been reported.\textsuperscript{45}

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Recent evidence suggests that mild or moderate hypothermia (32°C to 33°C) improves the clinical outcome in patients with traumatic brain injury.1 Initial studies have revived interest in the use of mild hypothermia in patients with ischemic stroke.2 However, the temporal window of efficacy and the duration of hypothermia that result in long-lasting protection have not been defined. In the accompanying article, Yanamoto and colleagues demonstrate, in a rat model of transient focal cerebral ischemia, that the hypothermic regimen that provides the best protection consists of a combination of intraischemic and prolonged (22 hours) postischemic hypothermia (33°C). Thus, to be effective, moderate hypothermia has to be present at the time of the onset of ischemia and has to be continued in the extended postischemic period. The present preclinical results have important implications for clinical studies on the therapeutic effect of hypothermia in stroke patients. For example, the findings suggest that hypothermia has to be instituted early after the onset of ischemia. Therefore, future studies should make an effort to lower body temperature early in the course of the stroke. Furthermore, long-lasting protection is observed only when the temperature is lowered for a prolonged period of time. Prolonged hypothermia is likely to increase the risk of complications, such as pneumonia, that have been observed in stroke patients in whom brain temperature was lowered for 2 to 3 days.2

There are other factors that need to be considered. In patients with MCA occlusion and increased intracranial pressure, hypothermia (33°C) for 2 to 3 days after stroke reduced intracranial pressure.2 However, 11 of 25 patients (44%) herniated and died of massive cerebral edema that developed during the rewarming period.2 This is a serious complication that may prove to be an obstacle to the use of hypothermia in patients with ischemic stroke.

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