Prolonged Mild Hypothermia Therapy Protects the Brain Against Permanent Focal Ischemia

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**Background and Purpose**—The efficacy of hypothermic intervention for permanent focal ischemia has yet to be clarified. This study investigated the effect of a prolonged moderate or mild hypothermia on permanent focal ischemia in rats.

**Methods**—Two permanent focal ischemia models in male Sprague-Dawley rats were used. Moderate (30°C, in experiment 1) or mild (33°C, in experiment 2) hypothermia was achieved at the time of the induction of focal ischemia and was maintained for 2 hours under general anesthesia. Thereafter, the hypothermic condition was maintained by means of a cold room for a total of 24 hours. The infarct volume and neurological function were analyzed for a maximum of 21 days and compared with that of the normothermia group. Regional cerebral blood flow was monitored for 6 hours in the ischemic core and penumbra region.

**Results**—In experiment 1, the total infarct volume in the normothermic group was 368±59 mm³; in contrast, it was significantly smaller in the hypothermia group: 169±33 mm³ at 48 hours (mean±SEM, \(P<0.05\)). In experiment 2, the infarct volume was 211±19 mm³ in the normothermia group and 88±15 mm³ in the hypothermia group at 21 days (\(P<0.05\)). There were significant differences in neurological function from days 2 through 21 between the two groups. Mean regional cerebral blood flow in the penumbra region increased to a level >50% of baseline.

**Conclusions**—Prolonged mild hypothermia suppressed the development of cerebral infarct and neurological deficit chronically after the induction of permanent focal ischemia. (Stroke. 2001;32:232-239.)

**Key Words:** animal models ■ cerebral blood flow ■ cerebral infarction ■ cerebral ischemia ■ hypothermia

Most cases of human ischemic stroke are caused by irreversible permanent occlusion of cerebral arteries. To recover the critically reduced regional cerebral blood flow (rCBF), thromboembolic intervention aimed at the revascularization of occluded sites has been demonstrated as a new clinical intervention for patients with ischemic stroke.\(^1\)-\(^5\) In addition to the endovascular thrombolytic approach, mild or moderate hypothermia therapy has been proposed as an intervention for cerebral ischemia, based on laboratory investigations in vivo.\(^6\)-\(^12\) Prolonged hypothermia is known to have several side effects;\(^13\) however, mild hypothermia (32°C) is much safer than the classic deep hypothermia.\(^14\)-\(^18\) We have recently reported a protocol providing potent and persistent neuroprotection against temporary focal ischemia in an experimental rat stroke model.\(^19\),\(^20\) Mild hypothermia therapy was most effective when it was applied both during and for a prolonged period after ischemia in the paradigm of a 2-hour, temporary focal ischemia. Although the neuroprotective effects of mild to moderate hypothermia therapy on global or focal temporary ischemia have been characterized and approved in many experimental protocols,\(^6\)-\(^8\),\(^10\),\(^21\)-\(^23\) the efficacy of hypothermia against permanent focal ischemia has been considered poor.\(^24\) Recently, clinical research showed that moderate hypothermia for 2 to 3 days initiated at 14 hours (on average) after the onset of ischemic symptoms improved clinical outcome (the mortality rate was reduced from 80% to 44%) in patients with severe permanent focal ischemia, that is, severe middle cerebral artery (MCA) occlusion.\(^25\) This was the first evidence of the efficacy of moderate hypothermia therapy for patients with severe ischemic stroke in the acute phase, including patients with irreversible focal ischemia who would later have permanent focal ischemia. Before conducting a large clinical trial of hypothermia therapy for patients with ischemic stroke, however, it is necessary to clarify the efficacy (or inefficacy) of mild or moderate hypothermia against irreversible severe focal ischemia in a well-controlled laboratory investigation. This study investigated the acute and delayed effect on infarct volume and neurological functions of prolonged mild or moderate hypothermia for treating severe permanent focal ischemia by using 2 sets of rat stroke models.
Materials and Methods

Experimental and Surgical Protocols

A total of 84 male Sprague-Dawley rats (SLC, Kyoto, Japan) (weight, 300 to 350 g) were used. 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.

To induce permanent focal ischemia in the neocortex, 2 different multivessel occlusion techniques were used. All the rats had access to food and water ad libitum until surgery. After an induction of general anesthesia with halothane (2%, in a gas mixture of oxygen and nitrogen), rats were ventilated mechanically through endotracheal intubation, and cannulation was performed in the right femoral artery to monitor mean arterial blood pressure (model AP-611G, AP-600G, Nihon Kohden), blood sugar, and blood gases (PO2, PCO2, pH; model ABL300, Radiometer Copenhagen). The mean arterial blood pressure was kept to within 100 to 120 mm Hg by adjusting the halothane concentration (0.5% to 1.0%). A digital thermometer was used to monitor rectal temperature beginning before the vessel occlusion; the temporal muscle temperature was simultaneously monitored with a digital thermometer.

Experiment 1

Under general anesthesia and after the monitoring of biological parameters, the bilateral carotid arteries were occluded just before the bifurcation with 5-0 silk sutures after a midline linear skin incision was made. The left temporal bone then was exposed and a small burr hole was made as described elsewhere.20 The left MCA was exposed after dural opening and permanently cauterized at the point of the rhinal fissure. After 3-vessel permanent occlusion had been achieved,26 the craniovascular wounds were closed, and the animal was kept under general anesthesia for 2 hours. Moderate (30°C) hypothermia was achieved at the time of the initiation of the 3-vessel occlusion by application of alcohol to the body surface and was maintained for 2 hours in the hypothermia groups. After surgery, the hypothermia was maintained by means of a cold room regulated at 6° to 8°C for 22 hours. The cold significantly prevented the elevation of body temperature and maintained hypothermia.23 The hypothermia was maintained by means of a cold room regulated at 6° to 8°C for 22 hours. The cold significantly prevented the elevation of body temperature and maintained hypothermia.23 The body temperature was monitored with a digital thermometer.

Measurement of rCBF

rCBF under the normothermic or hypothermic condition (the same range as in experiment 2) was monitored before and during focal ischemia with a laser-Doppler flowmetry (LDF, wavelength 780 nm) system (Laser Flow AMP, LFA2, Biomedical Science) in a separate set of 20 animals before and during ischemia of experiment 2. The regions of measurement were 1 mm caudal and 1 mm dorsal to the lateral border of the left MCA, which was the lateral border of the cerebral infarct (penumbra area), or 5 mm-distal to the point along the MCA artery (ischemic core area). In placing the probe, visible small vessels were avoided with the aid of a surgical microscope. The rCBF measurements were obtained just before and at 30 minutes, 1, and at every hour, for a total of 6 hours after cauterization of the MCA accompanied by simultaneous common carotid artery (CCA; right internal, left internal, and right external) occlusion.
Statistics
Physiological data (i.e., blood pressure, gases, blood sugar concentration, and pH), rCBF at each time point, infarct volumes, and infarct indexes were analyzed by ANOVA. The infarct volume, infarct index, edema ratio, and neurological deficit score at each time point were analyzed by 2-tailed 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 and rectal temperature were controlled within the physiological range (data not shown). There were no significant differences in such parameters among the groups.

Analyses of Cerebral Infarct

Experiment 1
The rectal (core) temperatures for 48 hours in all groups are shown in Figure 1. In the hypothermia group, the core temperatures were significantly lower for 24 hours ($P<0.05$). In the normothermia group, spontaneous hyperthermia (up to 39°C) was observed 6 to 8 hours from the induction of focal ischemia. The average core temperature was 5.2°C lower in the moderate hypothermic group than in the normothermic group during the initial 24 hours. Two rats died before the assessment on day 1 (mortality rate, 29%) and 4 rats died before the assessment on day 2 (mortality rate, 40%) in the normothermic control group. In contrast, in the hypothermia group, 1 rat died before day-1 assessment (mortality rate, 14%) and 2 rats died before day-2 assessment (mortality rate, 20%). The autopsy of these rats did not reveal any abnormality capable of causing death other than cerebral infarct. The total infarct volume (sum of neocortical and basal ganglia infarct volume) in the normothermic group was $306\pm26\text{ mm}^3$ at 24 hours and $368\pm59\text{ mm}^3$ at 48 hours (mean±SEM) (Table). In contrast, the total infarct volume in the hypothermic group was $136\pm15\text{ mm}^3$ at 24 hours and $169\pm35\text{ mm}^3$ at 48 hours, significantly smaller than that in the normothermic group at each time point ($P<0.05$). The significant differences observed in the infarct volume were also observed among the infarct indexes (Table) ($P<0.05$). Furthermore, the cerebral edema ratio was significantly smaller in the hypothermia than normothermia group ($P<0.05$).

Experiment 2
The core temperatures for 48 hours are shown in Figure 2. In the hypothermia group, the core temperature was significantly lower for the initial 14 hours ($P<0.05$). The average rectal temperature was 2.6°C—lower in the mild hypothermic group than the normothermia group during the initial 24 hours. Two rats died before the assessment on day 1 (mortality rate, 29%) and 4 rats died before the assessment on day 2 (mortality rate, 40%) in the normothermic control group. In contrast, in the hypothermia group, 1 rat died before day-1 assessment (mortality rate, 14%) and 2 rats died before day-2 assessment (mortality rate, 20%). The autopsy of these rats did not reveal any abnormality capable of causing death other than cerebral infarct. The total infarct volume (sum of neocortical and basal ganglia infarct volume) in the normothermic group was $306\pm26\text{ mm}^3$ at 24 hours and $368\pm59\text{ mm}^3$ at 48 hours (mean±SEM) (Table). In contrast, the total infarct volume in the hypothermic group was $136\pm15\text{ mm}^3$ at 24 hours and $169\pm35\text{ mm}^3$ at 48 hours, significantly smaller than that in the normothermic group at each time point ($P<0.05$). The significant differences observed in the infarct volume were also observed among the infarct indexes (Table) ($P<0.05$). Furthermore, the cerebral edema ratio was significantly smaller in the hypothermia than normothermia group ($P<0.05$).

### Cerebral Infarct Volume and Infarct Index (Without Edema) Values of Rats Subjected to Permanent Focal Neocortical Ischemia

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th></th>
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<th>Experiment 2</th>
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<td>Day 1</td>
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<tr>
<td></td>
<td>Normothermic</td>
<td>Hypothermic</td>
<td>Normothermic</td>
<td>Hypothermic</td>
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<td>Hypothermic</td>
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<tr>
<td>Total infarct volume, mm$^3$</td>
<td>306±26</td>
<td>136±15$^*$</td>
<td>368±59</td>
<td>169±35$^*$</td>
<td>139±21</td>
<td>77±20</td>
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<tr>
<td>(293±22)</td>
<td>(136±15)$^*$</td>
<td>(312±29)</td>
<td>(164±33)*</td>
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<tr>
<td>Edema ratio, %</td>
<td>11±1</td>
<td>6±1$^*$</td>
<td>20±3</td>
<td>7±2$^*$</td>
<td>10±1</td>
<td>5±2$^*$</td>
</tr>
<tr>
<td>Total infarct index, mm$^3$</td>
<td>276±24</td>
<td>118±13$^*$</td>
<td>347±55</td>
<td>158±33$^*$</td>
<td>127±21</td>
<td>73±19</td>
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<tr>
<td>(234±23)</td>
<td>(118±13)$^*$</td>
<td>(344±57)</td>
<td>(158±33)$^*$</td>
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</table>

Data are mean±SEM.

*Significant difference compared with value of the normothermic group ($P<0.05$).

Volume of neocortical infarct is shown in parentheses.
hours. No rat died before the assessment of cerebral infarct after ischemia. Twenty-one days after ischemia, the lesion of cerebral infarct, the white area in contrast to the red by TTC stain on day 2, was noted as a shrunken neocortex or a missing area in the neocortex (Figure 3, A and B). The average infarct volume for each group is shown in the Table. Although the size of the cerebral infarct was smaller in the hypothermia group on day 2, there was not a significant difference between the two (Table). The edema ratio was significantly smaller in the hypothermia group than in the normothermia group on day 2 (P<0.05)(Table). In a comparison of infarct volume (for day 2) and index (for day 21) in the normothermia group, the infarct index on day 21 was significantly larger than that on day 2 (Table). The cerebral infarct enlarged from days 2 to 21.

In terms of the measurement of infarct (missing lesion) volume in the chronic phase, the volume of the nonischemic hemisphere used for estimating the volume of normal brain was 667±25 mm³ (average±SEM, n=6), which was almost the same as the age-matched normal volume of the right hemisphere: 670±25 mm³ (average±SEM, n=6). It was demonstrated that there was no atrophy or enlargement in the right hemisphere on day 21 after ischemia in the left hemisphere in this model. Otherwise, the method might have underestimated or overestimated the actual infarct volume.

By cresyl-violet and GFAP stain, the lesion of cerebral infarct was again noted as a shrunken neocortex or a missing area in the neocortex (Figure 4). In the border of the lesion after necrosis, GFAP-positive reactive astrocytes and a thin layer of glial proliferation were observed (Figure 4, upper). The infarct index obtained by cresyl-violet stain, corrected by the shrinkage rate caused by the dehydration procedure in the staining (1.41 on average), was 186±15 mm³ in the normothermia group, whereas the total infarct volume in the hypothermia group was 96±17 mm³ (mean±SEM, n=6), which was again significantly smaller than that of the normothermia group (P<0.05).

Analyses of Cerebral Function
Severe neurological deficits were observed in the acute to subacute phases after the permanent focal ischemia in the
normothermia group (Figure 5). In contrast, in the hypothermia group, neurological deficits were mild throughout the observation period. There was a significant difference in the scores for each time point between the groups for the 3 weeks after the initiation of the permanent ischemia ($P < 0.05$).

There were similar tendencies of gradual recovery in both groups during the observation period.

**rCBF Measurement**

The results of the rCBF measurement in the ischemic core and penumbra are shown in Figure 6. A (normothermia group) and B (hypothermia group). In the normothermia group, rCBF increased in both the ischemic core and penumbra 3 hours after the induction of ischemia (Figure 6A). Under the hypothermic condition, the initial level of ischemia did not differ from that under the normothermic condition. However, the relief of ischemia was less prominent during this period. In both groups, the reduced rCBF recovered to a level of $>50\%$ of the preischemic baseline value in the penumbra and to $>30\%$ in the core region.

**Discussion**

It is reported that a surgical occlusion at the MCA origin (1 to 2 mm) caused cerebral infarct in only 13% of Sprague-Dawley rats used. By extending the coagulation site of the MCA trunk (2.5 mm from the orifice to the olfactory tract), a consistent but small neocortical infarct ($52 \pm 8.7$ mm$^3$, mean $\pm$ SD) was achieved in Sprague-Dawley rats. In contrast, a technique that used tandem permanent occlusion (distal MCA and ipsilateral CCA) produced a cerebral infarct that was larger but variable in size ($5 to 209 \pm 4 to 53 and 38 to 110 \pm 15 to 26$ mm$^3$, average $\pm$ SEM) in Wistar and Fisher 344 rats. The large variability in cerebral infarct volume on tandem occlusion was confirmed in our preliminary study with the same Sprague-Dawley rats (data not shown). To induce a consistent and large neocortical infarct, the temporary 3-vessel occlusion model (simultaneous occlusion of both CCAs and the unilateral MCA distal to the lenticulostriate branches) had been established. In the cerebral blood flow study, the cortical cerebral blood flow after 3-vessel occlusion reached 18%, from 48% by 2-tandem-vessel occlusion, or from 62% by a single distal MCA occlusion, which seldom induces infarct lesion. Regarding the variability between Wistar and Sprague-Dawley rats, it was reportedly larger in Wistar rats in cortical infarct when 3-vessel occlusion, including distal MCA occlusion, was used. The temporary 3-vessel occlusion technique causes consistent ischemia in the neocortex; however, when the occlusion was permanent, as in experiment 1, a significant mortality rate accompanied it in Sprague-Dawley rats. It has been reported that the intraluminal thread-occlusion model also had a high mortality rate in Sprague-Dawley rats (50% at 48 hours). To study the long term-outcome after permanent focal ischemia, it was necessary to significantly reduce mortality rates while retaining a consistent large neocortical infarction. After many trials, we have succeeded in developing a new permanent occlusion model, in which rats survive for a long period with moderate to large, consistent neocortical infarct.

Surprisingly, the volume of the infarct continued to grow from 1 (276 mm$^3$ on average) to 2 days (347 mm$^3$ on average)
Figure 6. rCBF values for ischemic core and penumbra region after induction of permanent focal ischemia under normothermic (A) or hypothermic (B) condition, with method of experiment 2. A, rCBF increased 3 hours after induction of ischemia. Four hours after induction of ischemia, mean rCBF was >50% of baseline value at penumbra region, a level considered safe for neurons. B, Mild hypothermia did not reduce rCBF further in either ischemic core or penumbra region compared with that under normothermic condition (A). However, relief of ischemia during the period was less prominent. Three hours after induction of ischemia, mean rCBF was >50% of baseline value at penumbra region.

There is evidence to suggest that mild hypothermia therapy protects the brain from temporary focal ischemia. In contrast, inefficacy of mild hypothermia therapy against permanent focal ischemia was reported. In that study, however, mild hypothermia was used only for a brief period, the initial 2 hours after the induction of ischemia. In another experiment, moderate hypothermia (29°C) for 2 days did not reduce the size of the cerebral infarct in a permanent focal ischemia model in monkeys. In that experiment, no animal survived for >5 days after ischemia, the time at which the cerebral infarct volume was analyzed. Regarding the high mortality rate in the acute phase, it is possible that a 2-day, continuous moderate hypothermia accompanied by general anesthesia affected the cardiopulmonary functions, resulting in deterioration in the animal’s general condition. In the present study, mild hypothermia significantly suppressed the development of cerebral infarct on permanent focal ischemia. A long-term–favorable outcome in neurological function as well as constructive preservation was demonstrated. Importantly, we used a prolonged period of mild hypothermia, extending to 24 hours after ischemia. Shorter hypothermia therapy, 1 hour to 12 hours, had been reported to be less effective than prolonged, 24-hour hypothermia in the temporary global or temporary focal ischemia in rats. Although the reduced cerebral blood flow was fully recovered at the end of temporary ischemia, a much longer period of hypothermic intervention was necessary to achieve a better outcome. In line with this, it is speculated that a short and mild hypothermia therapy is less effective against permanent focal ischemia because ischemia is more severe in permanent than in temporary ischemia in general. As for the degree of hypothermia, it is possible that a lower temperature better prevents cerebral ischemic injury; however, it is difficult to regulate the biological parameters in a physiological range during anesthesia under moderate to deep hypothermia in small animals. It has been reported that intraischemic moderate hypothermia (30°C) was less effective than intraischemic mild (33°C) hypothermia, possibly because of respiratory dysfunction (could be monitored by blood pH) during moderate hypothermic anesthesia.

The efficacy of neuroprotection continued on termination of the hypothermia therapy; that is, an acute and term-limited (1 day) application of mild hypothermia protected the brain from “permanent focal ischemia.” The therapy would be ineffective when the rCBF drops below the level critical for neurons to survive, and the situation lasts longer than the duration of hypothermia therapy. The unexpected positive results indicated that the duration of the severe drop in rCBF lasts <24 hours in the penumbra area in this model. In connection with this, it was demonstrated that the reduction of rCBF was not permanent but was recovering after the induction of permanent vessel occlusion, probably through the development of collateral flow. It was first demonstrated that “permanent focal ischemia” is not always accompanied by permanent reduction of rCBF to the level critical to neuronal survival.

Regardless of the improvement of rCBF through development of collaterals, which is considered favorable for brain tissue, delayed progression of the infarct lesion in the acute 2 days.
to 2 days) and the subacute (2 to 21 days) phases was demonstrated. The mechanism of chronic neuroprotection by hypothermia remains unknown; however, the significant suppression of cerebral edema formation in the acute phase might contribute to the prevention of the enlarging cerebral infarct, as seen in the present model. It is possible that cerebral edema, formed in and around the ischemic core, directly or indirectly acted to reduce the “recovered” rCBF of the peri-infarct area, with subsequent expansion of intravascular thrombosis, and acted to enlarge the infarct area after permanent vessel occlusion.

Recent reports state that the window of opportunity for induction of reperfusion after severe focal ischemia is brief, only 3 to 6 hours, in thrombolytic intervention for human ischemic stroke.3,4 Furthermore, it was speculated that the ratio of hemorrhagic transformation in brain lesions would increase if the thrombolytic intervention was initiated at a later time (>6 hours). However, the window for induction of mild hypothermia therapy appears to be larger. A recent clinical trial demonstrated that mild (33°C) hypothermia therapy for 2 to 3 days was effective for severe cerebral infarction caused by MCA occlusion in reducing the mortality rate (from 78% to 44%) when introduced 14 hours (in average) after the onset of ischemic symptoms.29 The existence of a delayed increase in infarct volume after permanent vessel occlusion, which has provided new insight into the pathophysiology of permanent ischemia, may explain in part why such delayed induction of hypothermia therapy induced a better outcome for patients with severe ischemic stroke.

In summary, the infarct volume increased in both the acute and subacute phases after irreversible vessel occlusion. Prolonged mild hypothermia therapy suppressed the extension of infarct lesion chronically, with the assistance of a spontaneous increase in rCBF. Given the present results, prolonged mild hypothermia therapy is potentially a powerful intervention for ischemic stroke triggered by sudden irreversible occlusion of cerebral vessels. The design of a mild hypothermia for acute ischemic stroke with minimum side effects in humans, as well as a controlled clinical trial, are the next steps for transient global ischemia. J Neurosci. 1990;10:311–316.


This study investigates the effect of hypothermia on cerebral ischemic damage. The authors demonstrate that in rat models of permanent focal ischemia, mild hypothermia induces a seemingly permanent reduction in brain damage. Furthermore, qualitative evaluation of cerebral blood flow suggests that the ischemic penumbra may be better perfused in hypothermic than in normothermic animals. The findings suggest that mild hypothermia is also beneficial in permanent focal ischemia and that hemodynamic factors may play a role in the mechanisms of the protection. However, to confirm the suggestion that the protection is flow related, the data obtained with laser-Doppler flowmetry need to be extended with absolute flow measurements in the region of the ischemic penumbra. Of interest is the observation that the area of lesion continues to expand for several days after the insult. This finding supports the emerging concept that ischemic damage progresses at a pace slower than previously believed and that the window of opportunity extends beyond the first few hours after ischemia. In this context, it would be useful to determine the therapeutic window for hypothermia in this model. This information would be valuable in planning hypothermia-based strategies to treat ischemic stroke.

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