Mild Hypothermia Ameliorates Ubiquitin Synthesis and Prevents Delayed Neuronal Death in the Gerbil Hippocampus

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Background and Purpose: The purpose of the present study is to determine the effect of mild hypothermia on the synthesis of ubiquitin, an important protein for maintenance of cell viability, in the hippocampal neurons following transient cerebral ischemia.

Methods: Transient ischemia was induced by occluding both common carotid arteries for 5 minutes. In experiment 1, the animals were divided into four groups according to the rectal and scalp temperatures during ischemia: the normothermia group and the graded hypothermia A, B, and C groups (n=9 per group). CA1 neuronal density was assessed at 7 days after ischemia. In experiment 2, the animals were divided into two groups designated the normothermia and the hypothermia groups (n=6 per group). The presence of ubiquitin was examined by immunohistochemistry at 6, 24, and 48 hours after transient ischemia in various regions of the hippocampus.

Results: In experiment 1, the mean±SEM neuronal density per millimeter was 12±1 in the normothermia group and 126±25, 225±10, and 214±9 in hypothermia groups A, B, and C, respectively. Mild hypothermia in groups B and C, in which the brain temperature was below 33°C, ameliorated markedly the extent of ischemic neuronal damage in the CA1 sector (p<0.01). In experiment 2, ubiquitin immunoreactivity had disappeared in all regions of the hippocampus at 6 hours after ischemia and showed no subsequent recovery in the CA1 pyramidal neurons under normothermic conditions. Under hypothermic conditions, however, it had recovered significantly in the CA1 pyramidal neurons at 24 and 48 hours after ischemia (p<0.01).

Conclusions: We conclude that mild hypothermia, in which the brain temperature is below 33°C, markedly improves the ischemic delayed neuronal damage in the CA1 sector, and that increased ubiquitin synthesis and protein ubiquitination could be one essential part of the protective mechanism afforded by mild hypothermia against delayed neuronal death.

Since Kirino1 reported delayed neuronal death in the hippocampal CA1 sector following transient cerebral ischemia, various mechanisms have been proposed to explain this phenomenon. Among these, an elevation of extracellular glutamate concentration during ischemia and impairment of protein synthesis in the pyramidal neurons after ischemia are considered to play a key role,2,3 although the relationship between them has not yet been clarified. In 1987, Busto et al4 reported that a reduction in the temperature of the brain by 2°C during forebrain ischemia significantly diminished the extent of neuronal injury in the CA1 sector of the rat and that this was associated with a marked reduction in the extracellular release of glutamate during ischemia.5 However, the effect of mild hypothermia on protein synthesis in the pyramidal neurons after ischemia has not been investigated previously.

In the present study, we examined the effect of graded mild hypothermia on the degree of CA1 ischemic neuronal injury in a gerbil model to determine the optimum temperature for exerting a protective effect and also compared protein synthesis in the hippocampal neurons after transient ischemia under normothermic and markedly protective hypothermic conditions using immunohistochemistry. We examined ubiquitin, a small protein found in all eukaryotic cells, as a representative intracellular protein. It binds covalently to short-lived proteins and

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abnormal proteins produced by various forms of injury, that is, designated as ubiquitination, after which the ubiquitinated proteins are digested by protease. Therefore, loss of ubiquitin leads to an accumulation of abnormal proteins, which may affect cellular structure and function and eventually cause cell death. Ubiquitin is thus an indispensable protein for intracellular repair and maintenance of cell viability after ischemia.

Materials and Methods

Experiment 1

Adult male Mongolian gerbils, weighing 60–80 g and obtained from Seiwa Experimental Animals, Japan, were used in this study. The animals were given free access to food and water before surgery, and then anesthetized with 2.5% halothane in 70% N2O and 30% O2. Both common carotid arteries were exposed through a midline cervical incision, and bilateral forebrain ischemia was induced 1 minute after discontinuation of halothane by occluding the common carotid arteries with aneurysm clips for 5 minutes. After ischemia, the clips were removed and restoration of carotid blood flow was verified visually. The skin incision was sutured under anesthesia with 2% halothane, and the animals were moved to a warm cage after discontinuation of anesthesia and given free access to food and water until histopathological examinations were performed.

Body core and head temperatures were monitored with rectal and scalp sensors, rather than with thermal probes implanted in the hippocampal parenchyma, to avoid mechanical injury, and the animals were divided into four groups according to the desired temperature during ischemia: normothermia group, defined as having a rectal temperature of 37°C and scalp temperature of 36.3°C; hypothermia A group, rectal temperature 35.5°C and scalp temperature 34.8°C; hypothermia B group, rectal temperature 34.5°C and scalp temperature 33.8°C; hypothermia C group, rectal temperature 33.5°C and scalp temperature 32.8°C. There were nine gerbils in each group. In the hypothermia groups, the desired degree of hypothermia was attained 10 minutes before cerebral ischemia by lowering the rectal and scalp temperatures, and the same temperatures were maintained within 0.5°C during ischemia. Normothermia was restored within 10 minutes after recirculation, after which the anesthesia was discontinued. In the normothermia group, the normal body temperature was maintained within 0.5°C throughout the experiment. Cooling and warming of the gerbils were achieved using a small cooling fan and a warming fan in combination with an overhead lamp, respectively, which were directed evenly over the body from the head to the hip.

In another experiment, correlations between rectal temperature (X) and scalp temperature (Y), between rectal temperature (X′) and brain (hippocampal) temperature (Y′), and between scalp temperature (X″) and brain temperature (Y″) were examined within a range of 33°C to 38°C of a rectal temperature in four gerbils. The scalp temperature was monitored at the bregma point, and the hippocampal temperature was monitored at a point 2 mm lateral from the midline, 1.5 mm caudal to the bregma, and 1.5 mm deep from the cortical surface. Each temperature response was related predictably and reliably to every other temperature response, and the respective regression lines and correlation coefficients from 40 measurements, 10 random measurements from each animal, were represented by the following equations and values: Y = −0.33+0.99X, r = 0.99; Y′ = 6.83+0.75X′, r = 0.99; Y″ = 8.34+0.72X″, r = 0.98. These correlations were all statistically significant (p < 0.01) (Figures 1a, 1b, and 1c).

For histopathological examination of the tissue, the brain was removed from each gerbil in all groups at 7 days after ischemia. The gerbils under 2% halothane anesthesia were perfusion-fixed with saline followed by 10% formaldehyde by transcerebral perfusion at a pressure of 120 cm H2O. After removal, the brains were dehydrated through a graded ethanol series, soaked in xylene, and embedded in paraffin. Coronal sections 6 μm thick of the dorsal hippocampus between 1.4 mm and 1.9 mm caudal to the bregma were prepared and stained with hematoxylin and eosin. The neuronal density in the right and left hippocampal CA1 sectors, that is, the number of nondamaged CA1 neurons per 1 mm linear length of the stratum pyramidale, was then determined in one section from each gerbil, since similar neuronal damage occurs throughout the rostrocaudal extent of the dorsal hippocampus. In five normal gerbils, the neuronal density in the CA1 pyramidal cell layer was also assessed. Data for each group were expressed as mean±SEM, and statistical analysis was performed using one-way analysis of variance and the Scheffe method; differences were considered significant at p < 0.05.

Experiment 2

Transient forebrain ischemia was induced in the same manner. The animals were divided into two groups according to the desired temperature during ischemia: normothermia group, rectal temperature 37°C and scalp temperature 36.3°C; hypothermia group, rectal temperature 34°C and scalp temperature 33.3°C; n = 6 in both groups. The body temperatures were maintained at the desired levels within 0.5°C during ischemia in both groups, and the procedures for regulating the temperature of the animals were the same as described for experiment 1.

For immunohistochemical study of the tissue, the gerbil brains were removed at 6 (n = 6), 24 (n = 6), and 48 (n = 6) hours after ischemia. The gerbils under 2% halothane anesthesia were perfusion-fixed with saline followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, by transcerebral perfusion at a pressure of 120 cm H2O. After removal, the brains were placed in perfusion solution at 4°C overnight. After fixation, the brains were dehy-
Correlations between rectal and scalp temperatures (panel a), rectal and brain (hippocampal) temperatures (panel b), and scalp and brain temperatures (panel c). T., temperature.

Experiment 1

The mean±SEM neuronal cell density in the CA1 sector in five normal gerbils was 254±4/mm. In the nine gerbils subjected to 5 minutes of bilateral forebrain ischemia under normothermic conditions, extensive neuronal damage was observed in the CA1 sector; the density of intact neurons was 12±1/mm (Table 1, Figure 3a). The CA1 pyramidal neurons were well preserved under hypothermic conditions. The neuronal cell density in the hypothermia A, B, and C groups was 126±24/mm, 225±10/mm, and 214±9/mm, respectively (Table 1), and these values were significantly higher than that in the normothermia group (p<0.01). Moreover, mild hypothermia in groups B and C with a rectal temperature below 35°C, at which the brain temperature is below 33°C, ameliorated markedly the extent of ischemic neuronal damage in the CA1 sector (Figure 3b). No significant difference was recognized between hypothermia groups B and C. In none of the gerbils subjected to transient forebrain ischemia was any

dratered through a graded ethanol series, soaked in xylene, and embedded in paraffin. Coronal sections 4 μm thick of the dorsal hippocampus between 1.4 mm and 1.9 mm caudal to the bregma were prepared, and ubiquitin immunoreactivity was examined in one section from each gerbil. A monoclonal antibody against ubiquitin was obtained from Chemicon International Inc. and was used at a dilution of 1:100. Sections of brain were deparaffinized, rinsed in phosphate-buffered saline, and pretreated with 0.3% hydrogen peroxide. The sections were then incubated with primary antibody solution for 12 hours at 4°C. This was followed by incubation with biotinylated anti-immunoglobulin in phosphate-buffered saline with normal serum, carrier protein, and a preservative (Biogenex Laboratories) for 60 minutes, and with horseradish peroxidase–conjugated streptavidin in phosphate-buffered saline with carrier protein and a preservative (Biogenex Laboratories) for 60 minutes. Each incubation was performed at room temperature. After a final wash, horseradish peroxidase binding sites were detected in 0.02% diaminobenzidine tetrahydrochloride and 0.05 M Tris buffer containing 0.005% H₂O₂. In five normal gerbils, the same immunohistochemical staining for ubiquitin was performed. Ubiquitin immunoreactivity was assessed in various regions of the hippocampus according to one of the following four grades: grade 0, no neurons positive; grade 1, only a few neurons positive (0–30%); grade 2, large number of neurons positive (30–70%); grade 3, most or all neurons positive (70–100%). Statistical analysis was performed using Fisher's exact probability test; differences at p<0.05 were considered significant.
marked difference of neuronal damage observed between the right and left sides of the hippocampal CA1 sector.

Experiment 2
In normal gerbils, ubiquitin immunoreactivity (grade 3 staining) was recognized in all neurons in various regions of the dorsal hippocampus. The cell nucleus, cytoplasm, and dendrites were all well stained. Figure 2 summarizes ubiquitin immunoreactivity in various regions of the hippocampus after transient forebrain ischemia under normothermic and hypothermic conditions.

In the gerbils subjected to transient forebrain ischemia under normothermic conditions, ubiquitin immunoreactivity had disappeared (grade 0 staining) in all regions of the hippocampus in all gerbils at 6 hours after ischemia. It had recovered only in neurons of the CA3 sector and the dentate gyrus at 24 and 48 hours after ischemia. However, in neurons of the CA1 sector, which are vulnerable to ischemia, ubiquitin immunoreactivity never recovered at any time point after ischemia (Figure 4a); grade 0 staining was observed in all gerbils except one in which only a few neurons of the CA1 sector showed ubiquitin immunoreactivity at 48 hours after ischemia. In the interneurons of the CA1 stratum radiatum, ubiquitin immunoreactivity was maintained throughout the postischemic period.

Under hypothermic conditions, major changes in ubiquitin immunoreactivity after transient ischemia were observed. Ubiquitin immunoreactivity in the CA1 sector had disappeared at 6 hours after ischemia, as under normothermic conditions; grade 0 staining was observed in all gerbils. However, it had recovered significantly at 24 hours and almost completely at 48 hours after ischemia (p<0.01) (Figure 4b), with grade 3 staining observed in all gerbils at 48 hours. The immunoreactivity also recovered in the nucleus, cytoplasm, and dendrites (Figure 4c). These findings were in contrast to those under normothermic conditions. Ubiquitin immunoreactivity in the CA3 sector and the dentate gyrus under hypothermic conditions was similar to that under normothermic conditions, although the immunoreactivity at 6 hours after ischemia was less impaired; grade 2 staining was present in all gerbils. In none of the gerbils subjected to 5 minutes of transient forebrain ischemia was any marked difference in ubiquitin immunoreactivity observed between the right and left sides of the hippocampus, and changes in ubiquitin immunoreactivity in the CA3 sector and the dentate gyrus were similar at all three time points after ischemia, under both normothermic and hypothermic conditions. Sections incubated with nonimmunized immunoglobulin G showed no significant staining.

Discussion
Ubiquitin, a small protein consisting of 76 amino acids with a molecular mass of 8.5 kd, is present in all eukaryotic cells and has some functions essential for cell viability. It is involved in chromosomal function in the cell nucleus, thus playing a role in regulation of the cell cycle. Proteins with a high turnover rate, such as regulatory proteins, and abnormal proteins produced by various forms of injury are discriminated and conjugated by ubiquitin in an ATP-dependent manner, after which ubiquitinated proteins are digested by protease. In neurons, this participation of ubiquitin in energy-dependent protein breakdown is important for disposal of high turnover and abnormal proteins produced by hypoxia, ischemia, or heat exposure. In this respect, ubiquitin is indispensable for neuronal restoration after transient ischemia, because loss of ubiquitin leads to an accumulation of high turnover and degenerated proteins, which could result in the formation of protein aggregates, affecting intracellular structure and function and eventually leading to cell death. The present study has demonstrated that ubiquitin disappeared and did

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**Table 1. Neuronal Cell Density in the CA1 Pyramidal Cell Layer 7 Days After Ischemia at Various Body Temperatures**

<table>
<thead>
<tr>
<th>Group</th>
<th>Temperature</th>
<th>Rectal</th>
<th>Scalp</th>
<th>n</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated normal</td>
<td>37.0°C 36.2°C</td>
<td>9</td>
<td>12±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient forebrain ischemia Normothermia</td>
<td>35.5°C 34.7°C</td>
<td>9</td>
<td>126±25*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothermia A</td>
<td>34.5°C 33.7°C</td>
<td>9</td>
<td>225±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothermia B</td>
<td>33.5°C 32.7°C</td>
<td>9</td>
<td>214±9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothermia C</td>
<td>33.5°C 32.7°C</td>
<td>9</td>
<td>214±9*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM cells per 1 mm linear length. NS, statistically not significant.

*p<0.01 compared with normothermia group.
†p<0.01 compared with hypothermia A group.

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**Figure 2. Ubiquitin (Ub) immunoreactivity in the hippocampal CA1 and CA3 sectors and the dentate gyrus at 6, 24, and 48 hours after transient forebrain ischemia under normothermic and hypothermic conditions.** Ub immunoreactivity in the CA1 sector recovered significantly under hypothermic conditions at 24 and 48 hours after ischemia. ○, Normothermia; ●, hypothermia. N=6 in each group; *p<0.01 significantly different from normothermia.
not recover after 5 minutes of transient ischemia in the CA1 pyramidal neurons of normothermic gerbils, and this has been proposed as one of the mechanisms causing delayed neuronal death.\textsuperscript{11}

Disappearance of ubiquitin without recovery in the CA1 pyramidal neurons after transient ischemia indicates that ubiquitin breakdown and persistent impairment of its synthesis occurred. Previous studies suggest that impairment of ubiquitin synthesis is the major factor responsible for its disappearance in the CA1 neurons after ischemia. Impairment of protein synthesis, including that of heat-shock protein, in the CA1 pyramidal neurons after transient ischemia, has been reported by Thilmann et al\textsuperscript{3} and Vass et al.\textsuperscript{12} The mechanism involved is not yet certain, but some possibilities have been proposed. Xie et al\textsuperscript{13} reported that the threshold for complete suppression of protein synthesis is much higher than that for maintenance of the tissue energy state. Therefore, impairment of protein synthesis must be due to minor alterations in cell homeostasis. One such change could be a slight decrease in intracellular pH, which declines before the decrease of ATP and the accumulation of lactate.\textsuperscript{14} It has been reported that minor acidosis reduces the rate of protein synthesis in vitro,\textsuperscript{15} and that tissue acidosis in the CA1 sector after transient ischemia continues for a longer period than in other brain regions.\textsuperscript{16}

Another change could be a slight shift in the activity of polypeptide chain initiation factor eIF2, which participates in the initiation of protein synthesis. The activity of this factor is influenced by the intracellular guanosine triphosphate:guanosine diphosphate ratio,\textsuperscript{17} which could be reduced by ischemia. Another possibility is ribosomal damage. Kirino\textsuperscript{18} reported that disaggregation of polyribosomes in the CA1 pyramidal neurons occurred immediately after reperfusion following transient ischemia. Normal protein synthesis would not be able to occur in such disaggregated ribosomes.\textsuperscript{19,20} The possibility of downregulation of protein synthesis at the transcriptional level is controversial. Maruno and Yanagihara\textsuperscript{21} found a positive correlation between loss of messenger RNA of cytoskeletal proteins after

**Figure 3.** The CA1 pyramidal cell layer 7 days after ischemia in gerbils under normothermic (panel a) and hypothermic (panel b) conditions. Neurons are well preserved under hypothermic conditions (groups B and C). Hematoxylin and eosin stain. Magnification, \(\times20\).
FIGURE 4. Immunohistochemical staining for ubiquitin in CA1 pyramidal neurons at 24 and 48 hours after ischemia. No neurons show immunoreactivity in the normothermia group; grade 0 (panel a). In contrast, ubiquitin immunoreactivity recovered in most of the neurons in the hypothermia group; grade 3 (panel b). The cell nucleus, cytoplasm, and apical dendrites are well stained in the hypothermia group (panel c). Cell nuclei were visualized by counterstaining with hematoxylin. Magnifications, ×20 (panels a and b) and ×40 (panel c).

transient ischemia and delayed neuronal death using the in situ hybridization method, whereas Nowak et al. found the prolonged expression of 70-kd heat-shock protein RNA in the CA1 pyramidal neurons after transient ischemia until the death of this cell population. Although the ubiquitin system is not fully elucidated, ubiquitin synthesis is believed to be impaired by the mechanisms described above.

It has been reported that mild hypothermia affords protection against delayed neuronal death in the
hippocampal CA1 sector after transient ischemia.\textsuperscript{5,23} In our present experiment, mild hypothermia was also found to ameliorate markedly delayed ischemic neuronal damage in the CA1 sector. Busto et al\textsuperscript{5} reported that an elevated concentration of excitatory amino acids in the extracellular space during ischemia, which causes calcium influx into the intracellular space via N-methyl-D-aspartate receptor-gated ion channel,\textsuperscript{24–26} was suppressed by mild cerebral hypothermia. However, changes in protein synthesis due to mild hypothermia in the CA1 pyramidal neurons after transient ischemia have not been investigated previously.

The present study, in which ubiquitin immunoreactivity that had disappeared at 6 hours after ischemia, recovered at 24 and 48 hours, suggests that synthesis of ubiquitin, an indispensable protein for neuronal viability, increased after transient ischemia with mild hypothermia in the CA1 pyramidal neurons, although it must be borne in mind that immunohistochemistry does not directly reflect protein synthesis. Protein synthesis as a whole in the CA1 pyramidal neurons also may be improved, although further investigations will be necessary to clarify the mechanism, possibly at the transcriptional level, by which mild hypothermia ameliorates the impaired protein synthesis.

In this study, mild hypothermia was established deliberately before ischemia induction and maintained throughout the ischemic period. It is believed that this type of mild hypothermia is a useful therapeutic tool in humans when transient ischemia is anticipated, such as temporary clipping of major cerebral arteries in neurosurgery and cerebral vasospasm after subarachnoid hemorrhage due to aneurysmal rupture.

In conclusion, protein ubiquitination in the CA1 pyramidal neurons after transient ischemia improved under mild hypothermic conditions, and this improvement assisted the smooth restoration of intracellular structure and function after ischemia, and lead to the prevention of delayed neuronal death. Improvement of protein ubiquitination by 48 hours after ischemia could be one essential part of the protective mechanism afforded by mild hypothermia, as delayed neuronal death in the CA1 sector occurs after 72 hours following ischemia.

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