Temperature Modulation of Ischemic Neuronal Death and Inhibition of Calcium/Calmodulin-Dependent Protein Kinase II in Gerbils

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We used brief bilateral carotid artery occlusion in gerbils to examine the effects of temperature on ischemia-induced inhibition of calcium/calmodulin-dependent protein kinase II activity and neuronal death. In normothermic (36°C) gerbils, ischemia induced a severe loss of hippocampal CA1 pyramidal neurons measured 7 days after ischemia (28.4 neurons/mm, n=10; control density in 10 naive gerbils 262.1 neurons/mm) and a significant decrease in forebrain calcium/calmodulin-dependent protein kinase II autophosphorylation measured 2 hours after ischemia (12.9 fmol/min, n=6; control phosphorylation in six naive gerbils 23.5 fmol/min). The effect of temperature on these indicators of ischemic damage was examined by adjusting intracerebral temperature before and during the ischemic insult. Hyperthermic (39°C) gerbils showed almost complete loss of neurons in the CA1 region (3.0 neurons/mm, n=11) and extension of neuronal death into the CA2, CA3, and CA4 regions. In addition, hyperthermia exacerbated ischemia-induced inhibition of calcium/calmodulin-dependent protein kinase II activity (4.2 fmol/min, n=6). Hypothermia (32°C) protected against ischemia-induced CA1 pyramidal cell damage (257.0 neurons/mm, n=20) and inhibition of calcium/calmodulin-dependent protein kinase II activity (26.0 fmol/min, n=6). Our results are consistent with the hypothesis that loss of calcium/calmodulin-dependent protein kinase II activity may be a critical event in the development of ischemia-induced cell death. (Stroke 1990;21:1715-1721)

Cerebral ischemia produces morphologic and functional modifications in neurons that ultimately can result in neuronal death.1-3 Although several studies investigating its pathologic mechanisms have provided important information, the specific molecular basis of ischemia-induced cell death remains unknown. Alteration of calcium homeostasis and calcium-dependent mechanisms have been implicated as phenomena that may be essential contributors to cell death caused by ischemia. Specifically, neuronal calcium-dependent protein phosphorylation has been shown to be very sensitive to the effects of ischemia.4,5 In gerbils, 5 minutes of forebrain ischemia induces an early and permanent inhibition of multifunctional calcium/calmodulin-dependent protein kinase II (CaM kinase II) activity.4 Since CaM kinase II mediates many of the second messenger effects of calcium, including neuronal excitability, synaptic modulation, cytoskeletal function, and neurotransmitter release,6-10 we investigated its role in the development of ischemia-induced cell death.

One useful strategy to investigate the possible relation between inhibition of CaM kinase II activity and the development of ischemia-induced neuronal death is to employ a condition that modulates the effect of ischemia on cell death and determine if this modulator also affects CaM kinase II activity. Hypothermia has been shown to prevent ischemic neuronal death in many animals,11-16 including gerbils.15 Conversely, hyperthermia has a detrimental effect on the recovery of metabolic function in cats subjected to global cerebral ischemia.17 Thus, temperature has been proven to modulate the extent of ischemia-induced death of neurons. We altered intras ischemic...
temperature to determine its effect on ischemia-induced inhibition of CaM kinase II activity.

Brief-duration bilateral carotid artery occlusion in gerbils has proven to be a useful model for studying the effects of ischemia on neuronal death.\textsuperscript{18-20} Five minutes of ischemia in this model produces almost complete destruction of pyramidal neurons in the hippocampal CA1 region within 7 days after ischemia.\textsuperscript{4,15,18-20} The delayed nature of ischemia-induced cell death in gerbils makes this species an ideal model for studying the biochemical changes occurring in ischemic neurons that may initiate the pathway leading to cell death. We have previously demonstrated that an early (<2 hours after ischemia) event following ischemic insult is the permanent loss of CaM kinase II activity.\textsuperscript{4} In this study, we employed the gerbil model of bilateral carotid artery occlusion to study the effects of temperature modulation on ischemia-induced pyramidal cell death and ischemia-induced inhibition of CaM kinase II activity.

Materials and Methods

One hundred eleven gerbils (Meriones unguiculatus) weighing 60-70 g were anesthetized with 0.4 ml/100 g i.p. equithesin, and guide cannulas were stereotactically implanted -1.7 mm rostrocaudally and 1.5 mm left and right of the bregma,\textsuperscript{21} 2 mm deep, 24 hours before the induction of ischemia. Placement of the guide cannulas into the dorsal hippocampus was checked by injecting dye and sectioning the brain of randomly selected test animals. On day 2, the gerbils were anesthetized with 2% halothane and an Omega hypodermic thermocouple (Stamford, Conn.) was inserted into each cannula. Intracerebral temperature was adjusted to the desired level by heating or cooling elements before the induction of ischemia and maintained throughout the ischemic period. Rectal temperatures were simultaneously monitored in all gerbils. Comparison of rectal and intracerebral temperatures during ischemia in this model demonstrated that rectal temperatures were consistently 0.5-1.5°C lower than the intracerebral temperature. Following ischemia, temperature adjustment was discontinued and all gerbils were allowed to gradually return to a normal body temperature. For the induction of ischemia, the gerbils were placed on a surgical table (anesthesia was maintained with 3% halothane) and both the left and right carotid arteries were exposed by a ventral cervical incision as described previously.\textsuperscript{4,15} Blood flow to the forebrain was halted for 5 minutes by bilateral carotid artery occlusion using Heifitz aneurysm clips. Cessation of blood flow, as well as post-ischemic restoration of blood flow, were confirmed visually using Zeiss prism loupes (×3.5 magnification) (Thornwood, N.Y.). The seven groups studied were unoperated naive animals, ischemic and sham-operated normothermic (36°C) animals, ischemic and sham-operated hyperthermic (39°C) animals, and ischemic and sham-operated hypothermic (32°C) animals.

For biochemical study, 42 gerbils were sacrificed by decapitation 2 hours after ischemia or sham operations. The forebrains were rapidly dissected and homogenized in ice-cold buffer as described previously.\textsuperscript{4} Forebrain homogenates were normalized for protein concentrations and studied for endogenous calcium-dependent protein phosphorylation. Standard phosphorylation reaction solutions contained 100 μg brain protein, 10 mM MgCl\textsubscript{2}, 7 μM \textsuperscript{32}P-adenosine triphosphate, 10 mM piperazine-N,N\textsuperscript{-}(2-ethanesulfonic acid) (pH=7.4), ±5 μM CaCl\textsubscript{2} and ±1 μg calmodulin. Standard reactions were performed in a shaking water bath at 37°C. They were initiated by the addition of Ca\textsuperscript{2+}, continued for 1 minute, and were terminated by the addition of 5% sodium dodecyl sulfate (SDS) stop solution. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (23 μg protein/lane). The gels were stained with Coomassie brilliant blue, dried, and exposed to x-ray film for autoradiography.\textsuperscript{4} Autophosphorylation of CaM kinase II was routinely assayed by analysis of the 50-kDa subunit of the enzyme, and the data are expressed as femtomoles phosphate incorporated into the 50-kDa band per minute quantified by scintillation counting of slices of the dried gel as well as by computer-assisted densitometry\textsuperscript{4} of the autoradiograph. Comigration with purified CaM kinase II on high-resolution SDS-PAGE was used to identify the quantified phosphoprotein as the 50-kDa subunit of CaM kinase II.

Protein samples from each group were also examined for overall patterns of calmodulin binding activity using the biotinylated calmodulin overlay method.\textsuperscript{22,23} Equal amounts of protein (100 μg) from naive, ischemic, and sham-operated brains were subjected to 10% SDS-PAGE, and resolved proteins were transferred to nitrocellulose and incubated with 10 μg/ml biotinylated calmodulin. Bound biotinylated calmodulin was detected using avidin/alkaline phosphatase and the BCIP/NBT Chromgen system.\textsuperscript{22,23}

In a parallel study, 69 gerbils were subjected to ischemia or sham operation as described above. After 7 days they were sacrificed and perfused, and their brains were processed for histologic examination as described previously.\textsuperscript{15} Coronal sections of brain were dehydrated in a series of ethanol and xylene baths before being embedded in paraffin. Serial sections 7 μm thick containing the dorsal hippocampus were cut using an American Optical AL microtome (Buffalo, N.Y.) and mounted on slides. The sections were stained with cresyl violet, and the density of viable CA1 and CA3 pyramidal neurons was quantified as number per millimeter using an American Optical microscope at ×400 magnification.

Results

In naive gerbils the pyramidal cell layer of the hippocampus was a well-defined band of neurons 4–6 cells thick with an average neuronal density of 262.1/mm in the CA1 region (Table 1). Five minutes of normothermic bilateral carotid artery occlusion caused
TABLE 1. Effect of Temperature on CA1 and CA3 Neuronal Density and CaM Kinase II Activity in Gerbils After 5 Minutes of Bilateral Carotid Artery Occlusion or Sham Operation

<table>
<thead>
<tr>
<th>Group</th>
<th>CA1 region</th>
<th>CaM kinase II activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Naive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothermia</td>
<td>10</td>
<td>262.1±3.4</td>
</tr>
<tr>
<td>Sham-operated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>20</td>
<td>257.0±4.2</td>
</tr>
<tr>
<td>Normothermia</td>
<td>6</td>
<td>252.7±4.5</td>
</tr>
<tr>
<td>Sham-operated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>11</td>
<td>248.8±5.4</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>6</td>
<td>28.4±11.9*</td>
</tr>
<tr>
<td>Sham-operated</td>
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</tr>
<tr>
<td>Ischemic</td>
<td>11</td>
<td>28.4±11.9*</td>
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table1: CaM kinase II, calcium/calmodulin-dependent protein kinase II; hypothermia, 32°C; normothermia, 36°C; hyperthermia, 39°C.

*p<0.001 different from sham-operated by Student’s t test.

significant death of pyramidal neurons 4–7 days later (Figure 1D), which was reflected in the significant decrease in the density of normal-staining neurons in the CA1 region of the dorsal hippocampus (Figure 2, Table 1). Sham-operated normothermic gerbils had a mean±SEM neuronal density of 250.2±6.9/mm in the CA1 region (Figure 1C, Table 1), suggesting that the surgery itself had no effect on cell death.

FIGURE 1. Photomicrographs of dorsal hippocampi of gerbils 7 days after either 5 minutes of bilateral carotid artery occlusion (B, D, and F) or sham operation (A, C, and E) under conditions of hypothermia (32°C) (A and B), normothermia (36°C) (C and D), or hyperthermia (39°C) (E and F). ×35 magnification.
Since decreased temperature has been shown to protect against ischemia-induced cell death in rats, we examined the effect of brain temperature on ischemia-induced pyramidal cell death in gerbils. Compared with the naive group, neither hypothermia nor hyperthermia had any significant effect on pyramidal cell density in the CA1 region of sham-operated animals (Figure 2, Table 1). In ischemic gerbils, decreasing brain temperature to 32°C resulted in complete protection from ischemia-induced cell death and average neuronal density did not differ from that in the sham-operated hypothermic group (Figure 1, A and B; Table 1). Increasing the brain temperature to 39°C in ischemic gerbils exacerbated cell death in the CA1 region as indicated by a significant decrease in neuronal cell density compared with the sham-operated hyperthermic group (Figure 1, E and F; Table 1). Thus, ischemia combined with increased temperature produced almost complete destruction of all neurons in the hippocampal subfields CA1-CA4 (Figure 1F). Taken together, the data indicate that not only are pyramidal neurons in the CA1 region of the dorsal hippocampus in gerbils extremely sensitive to the effects of ischemia, but also that the ischemic effect is profoundly influenced by intracerebral temperature.

Ischemia produced a significant decrease in CaM kinase II activity of the forebrain (Figure 3). Mean±SEM phosphate incorporation into the 50-kDa subunit of CaM kinase II from naive gerbils was 23.5±0.9 fmol/min (Figure 4). Sham-operated normothermic gerbils had a mean±SEM phosphate incorporation of 21.2±0.8 fmol/min (Table 1), indicating that surgery had no direct effect on CaM kinase II activity. Five minutes of bilateral carotid artery occlusion produced a significant decrease in CaM kinase II activity in ischemic normothermic animals (Table 1). Thus, ischemia significantly inhibited CaM kinase II activity measured 2 hours later.

We also examined the effects of temperature on ischemia-induced inhibition of CaM kinase II activity. Compared with the naive group, neither hypothermia nor hyperthermia caused any significant change in phosphorylation in sham-operated gerbils (Table 1), indicating that temperature itself had no direct effect on CaM kinase II activity. In ischemic animals, hypothermia protected against ischemia-induced inhibition of protein phosphorylation and the CaM kinase II activity level was not significantly
FIGURE 4. Bar graph of effect of 5 minutes of bilateral carotid artery occlusion at three temperatures on CaM kinase II activity in gerbils. Incorporation of PO₄ into 50-kDa subunit of CaM kinase II was quantified in preparations obtained 2 hours after either ischemia (shaded bars) or sham operation (filled bars). Data are expressed as mean±SEM (n=6 for each group). Open bar, unoperated naive gerbils. **p<0.001 different from sham-operated group by Student's t test.

different from those of the naive and sham-operated groups (Table 1). Hyperthermia intensified the ischemia-induced loss of CaM kinase II activity, further decreasing phosphate incorporation to 4.2±1.1 fmol/min (Table 1). Thus, forebrain CaM kinase II activity is extremely sensitive to the effects of ischemia and, like neuronal death, the ischemic effect is modulated by intracerebral temperature.

One possible explanation for alterations in CaM kinase II activity is destruction of the enzyme itself by mechanisms sensitive to the effects of ischemia and temperature, such as proteolytic digestion. To determine if decreased CaM kinase II activity was the result of enzyme degradation and a decrease in enzyme levels, brain homogenates used for enzyme reactions were resolved by high-resolution SDS-PAGE for direct quantification of CaM kinase II levels by protein staining. CaM kinase II is a multimeric protein with principal protein subunits of approximately 50 and 60 kDa. Examination of the Coomassie blue and more sensitive silver staining pattern of brain proteins showed that ischemia did not affect 50-kDa protein staining at any temperature studied (Figure 3A). High-resolution SDS-PAGE and comigration studies confirmed the identity of the enzyme subunits. Thus, it is not likely that extensive proteolytic effects on CaM kinase II are responsible for the ischemia-induced decrease in or the temperature modification of CaM kinase II activity.

An additional method of quantifying CaM kinase II subunit concentration is direct measurement of calmodulin binding to the protein subunits. Both the 50-kDa and 60-kDa subunits have calmodulin-binding properties. We noted prominent calmodulin-binding peptides at 36, 50, 60, 75, and 84 kDa in gerbil brain fractions. Comigration studies using purified CaM kinase II confirmed that the peptides at 50 and 60 kDa are the major protein subunits of CaM kinase II. No quantitative difference in biotinylated calmodulin binding to either the 50- or 60-kDa subunit of CaM kinase II was observed (Figure 5). Quantitative analysis of biotinylated calmodulin binding patterns in brain homogenates revealed no obvious differences between treatments for calmodulin binding to the 50-kDa subunit (Figure 5). The calmodulin binding studies provide strong evidence that the CaM kinase II subunit concentration is not affected by the experimental conditions employed and suggest that CaM kinase II is not a substrate of ischemia-induced proteolysis under these conditions. Thus, it is unlikely that the decline in calcium-dependent protein phosphorylation following an ischemic insult results from a direct alteration in calmodulin binding to the kinase; rather, it is likely that a posttranslational modification of the enzyme results in inactivation of CaM kinase II without a loss of calmodulin binding.

Discussion

Hippocampal pyramidal neurons, particularly those in the CA1 region of the dorsal hippocampus,
have long been known to be very sensitive to the effects of even mild cerebral ischemia. We demonstrate that the extent of ischemia-induced pyramidal cell damage in gerbils is significantly affected by intracerebral temperature. Hypothermia prevented ischemia-induced cell death. Lowering the brain temperature to 32°C resulted in complete protection of cell number and staining characteristics. A similar protective action of hypothermia against ischemic damage has been reported.11-16,24,25 Hyperthermia exacerbated ischemia-induced cell death. Raising the cerebral temperature to 39°C resulted in complete destruction of neurons in the CA1 region, with damage also observed in the less susceptible CA2, CA3, and CA4 regions. Thus, modifying cerebral temperature during ischemia profoundly altered the survival of hippocampal pyramidal neurons. Similarly, CaM kinase II activity is significantly affected by cerebral ischemia in this gerbil model.4 Hypothermia protected against and hyperthermia intensified the ischemia-induced inhibition of CaM kinase II activity. We demonstrate that the extent of ischemia-induced inhibition of CaM kinase II activity is affected by intracerebral temperature. Thus, lowering and raising the intracerebral temperature will decrease and increase, respectively, the extent of pyramidal cell damage and CaM kinase II inhibition caused by ischemia in gerbils.

Previous studies from this laboratory have shown that transient ischemia causes an early and permanent inhibition of CaM kinase II activity and suggested that the switch in activity state for this enzyme may be a causal factor in the development of neuronal death.4 It is important to establish whether the effects of ischemia on CaM kinase II activity represent an important trigger for mediating cell death or just an epiphenomenon of ischemic injury. To test if CaM kinase II effects are related to cell death, it would be useful to employ a condition that does not block ischemia but does affect cell damage and determine if the condition also prevents CaM kinase II inhibition. We used intracerebral temperature to regulate the degree of cell death caused by ischemia. Our results show a corresponding effect of temperature on ischemia-induced changes in CaM kinase II activity and cell death. Our results indicate that CaM kinase II activity may be directly related to cell viability and that hypothermia may exert some of its cerebroprotective actions through preservation of CaM kinase II activity.

CaM kinase II is an important mediator of the effects of transient increases in intracellular Ca²⁺ levels and is highly concentrated in the brain.6,28,29 Immunochemical evidence demonstrates that CaM kinase II is selectively expressed in neurons compared with glial cells and is highly concentrated in the pyramidal neurons of the hippocampus, comprising 2% of the total hippocampal protein.28,29 CaM kinase II has been implicated in both presynaptic and postsynaptic neuronal functions. Presynaptically, CaM kinase II has been shown to be involved in neurotransmitter release via phosphorylation of synapsin I and cytoskeletal elements.6-8,26,27 and injection of purified CaM kinase II into squid giant axon nerve terminals promotes neurotransmitter release.9 CaM kinase II is also concentrated postsynaptically and comprises 50% of the postsynaptic density protein.30-32 Thus, the significant alteration in CaM kinase II activity caused by ischemia could set into motion changes in ion fluxes, transmitter systems, cell transport mechanisms, and other calcium-regulated processes. These initial changes induced by a decrease in CaM kinase II activity could then initiate the gradual accumulation of ions, metabolic products, and toxins that eventually lead to cell death. Several other important biochemical processes are transiently altered by ischemia but return to preischemic levels following injury.2,23 The long-lasting changes seen in CaM kinase II activity suggest that this major calcium enzyme system is sensitive to ischemic injury and that long-lasting changes in calcium homeostasis that might occur through this modulation of CaM kinase II activity may underlie some of the initial events that trigger delayed cell death.

It is important to measure temperature by direct intracerebral methods since it has been shown that brain temperature may vary considerably from rectal temperature during ischemia.14 Therefore, all temperature measurements in this study were made with an Omega hypodermic thermocouple stereotactically implanted into the contralateral hippocampus to accurately measure hippocampal temperature during the ischemic insult. Because a drop of only 4°C can produce almost complete histologic protection, great caution must be taken in studies evaluating the effects of cerebroprotective agents or procedures to ensure that variation in intracerebral temperature is minimized. Thus, such factors as anesthetic, room temperature, temperature modification procedures, and the use of putative cerebroprotective agents (which may alter cerebral blood flow before and/or after ischemia) must be strictly controlled to properly evaluate their influence on ischemia-induced neuronal death.

The ischemia-induced decrease in CaM kinase II activity is an early, permanent event after 5 minutes of carotid artery occlusion and may represent an early index of ischemic damage.4 Studies of ischemia-induced inhibition of CaM kinase II activity in different forebrain regions show that it is decreased to a similar extent in all ischemic areas.4 To decrease the artifactual effect of dissecting out the hippocampus, we used forebrain homogenates to biochemically assess the effects of temperature and ischemia on CaM kinase II activity. The extent of ischemia-induced inhibition of CaM kinase II in normothermic gerbils is in agreement with data reported previously for forebrain and hippocampal inhibition.4 Experimental evidence indicates that hypothermia acts both presynaptically and postsynaptically in producing cerebroprotection. Busto et al.4 have shown that hypothermia protects against ischemia-induced excitatory neurotransmitter release in rat brain. Also,
the postsynaptic action of excitatory neurotransmitter antagonists such as MK-801 has a protective effect against ischemia-induced damage, and this effect is enhanced by decreasing brain temperature. It is unclear whether hypothermic protection of CaM kinase II is required presynaptically and/or postsynaptically to promote neuronal survival. However, the long-lasting significant decrease in neuronal CaM kinase II activity produced by ischemia most likely plays a major role in disrupting neuronal function and calcium signal transduction and suggests that some of the cytotoxic effects of ischemia may be mediated by alterations of this major calcium enzyme system.

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