Combination Therapy With Nimodipine and Dizocilpine in a Rat Model of Transient Forebrain Ischemia

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Background and Purpose: We explored the effectiveness of dual blockade of calcium channels in preventing ischemic necrosis in a rat model of transient forebrain ischemia.

Methods: To assess all the major brain regions, the entire brain was subserially sectioned and examined histologically 1 week after ischemia in 44 male Wistar rats. Brain temperature was monitored and controlled to avoid hypothermia or intergroup temperature differences at the time drugs were administered. All regimens were begun 20 minutes after the ischemia. Treated animals received either the L-type calcium channel blocker nimodipine (0.25 μg/min x 24-hour i.v. infusion), the noncompetitive N-methyl-D-aspartate receptor antagonist MK-801 (dizocilpine; 5 mg/kg i.v.), or both regimens in combination.

Results: In the neocortex (p<0.05) and striatum (p<0.05), only double-treated animals showed a statistically significant reduction in neuronal necrosis. Dual therapy eliminated neuronal necrosis in the caudate nucleus entirely. In the septal (densely ischemic) hippocampus, protection was weak and inconsistent (0.012<p<0.788), but in the temporal (incompletely ischemic) hippocampus, the dual-treated group showed the most significant reduction (p<0.006).

Conclusions: We conclude that the combination of nimodipine and MK-801, if begun 20 minutes after ischemia, may offer a neuroprotective effect against neuronal necrosis in transient forebrain ischemia and that protection is maximal in the major extrahippocampal brain regions. (Stroke 1992;23:725–732)

Key Words • calcium channel blockers • cerebral ischemia • neuronal damage • rats

Ischemia-induced release of excitatory amino acids and cellular calcium influx are held to be important factors in the pathogenesis of ischemic neuronal necrosis. Glutamate released into the brain extracellular space by ischemia activates several subtypes of excitatory amino acid receptors. One of these, selectively activated by N-methyl-D-aspartate (NMDA), plays a role in the pathophysiology of ischemic neuronal death. The NMDA receptor opens a cell membrane channel that is permeable to calcium, the opening of which causes significant increases in cytoplasmic calcium levels. In addition to cellular calcium entry through agonist-gated channels, NMDA receptor activation (or any depolarization) causes calcium influx through voltage-gated calcium channels. These channels are opened by a drop in the transmembrane voltage, are widely distributed throughout the brain, and, like NMDA-gated channels, are likewise capable of causing increases in cytosolic calcium levels.

Thus, cellular calcium entry in ischemia can occur by at least two mechanisms. Blockade of either mechanism seems a reasonable approach in treating ischemic cell death, and drugs are available which respectively block calcium influx through voltage-gated and agonist-gated calcium channels. Yet in various animal models of global brain ischemia, voltage-sensitive calcium channel antagonists and agonist-gated channel blockers, used individually, give either weak neuroprotection or no neuroprotective effect at all. We are thus left with a conundrum: ischemia leads to NMDA receptor activation and lethal neuronal calcium influx, yet drugs entering the brain and blocking calcium influx have a weak effect, if any effect at all. In this regard, it is well to consider more than one mechanism potentially leading to a rise in intracellular calcium. Attempts to block calcium entry by a single drug, acting on a single type of channel, may be doomed to be suboptimally or partially effective in anti-ischemic therapy. A combination of drugs blocking two routes of calcium entry might prove more effective.

Based on these concepts, we designed the present series of experiments to document the effect of dual therapy using two channel antagonists in transient forebrain ischemia. MK-801, or dizocilpine, was chosen because it is effective in penetrating the central nervous system and blocking NMDA-induced damage when given parenterally. The L-type voltage-sensitive calcium channel blocker nimodipine was chosen because of its demonstrated ability to reduce ischemia-induced influx of calcium into neurons. Drugs were administered singly or in combination. Because many experimental therapeutic studies in global types of ischemia have focused solely on the hippocampus, we examined the entire brain, subserially sectioned. The results sug-
gest that multiple routes of calcium entry should be considered in designing therapy aiming to metabolically protect central nervous system tissue in global types of ischemia.

**Materials and Methods**

Ischemia was induced for 10.5 minutes in 44 male Wistar rats weighing 272–455 g, using a modification of the previously described model of Smith and coworkers.15 Transient forebrain ischemia was induced by a combination of carotid clamping and hypotension. Rats were anesthetized in 3% halothane (MTC Pharmaceuticals, Mississauga, Canada), intubated, and ventilated on a Starling-type ventilator with a 2:1 N2O:O2 mixture containing 0.7% halothane. A tail vein was cannulated, and continuous infusion of suxamethonium chloride (2 mg/hr) was used to maintain paralysis during surgery. After a period of physiological stabilization of roughly 20 minutes, rats were given 5 mg/kg of the ganglion blocker trimethaphan (Arfonad, Hoffman-LaRoche, Toronto, Canada) intravenously to counteract the hypertensive response during carotid clamping. This allowed for maintenance of the intraischemic blood pressure at very close to 50 mm Hg in all groups (Table 1) by controlled exsanguination through the central venous line. During the subsequent drop in blood pressure, blood was withdrawn into the heparinized syringe to further lower the blood pressure to 50 mm Hg, at which time two Moria vessel clamps were placed on the carotid arteries. Blood pressure readings were automatically digitized every 6 seconds and recorded to computer disk. A central venous catheter was inserted into the right jugular vein and attached to a prewarmed heparinized syringe. Mean±SD body core temperature was maintained at 36.5±0.5°C with a servocontrolled homeothermic blanket control unit to provide a stable physical setting for the control of brain temperature. The latter was monitored and controlled separately. Because of the importance of intraischemic and postischemic brain temperature on neuronal necrosis and the effect of temperature on neuroprotection by MK-801,18,19 brain temperature was estimated by a thermocouple probe (Omega, Stamford, Conn.) inserted into the temporalis muscle. Head surface temperature was regulated to 35.5±0.5°C by an overhead incandescent lamp at all times except during the ischemic period, when it gradually dropped in all animals by 1.5°C to a nadir of 34±0.5°C just before reinfusion of the shed blood. We found in pilot experiments that to keep head surface temperature constant, it was necessary to lower the incandescent lamp over the head until it was almost physically touching the animal's fur. Because intraischemic cooling of the head due to bilateral carotid clamping takes place from the outside in, such measures were deemed redundant. Head surface temperature returned to control values within 2 minutes, and all treatment regimens were begun 20 minutes after ischemia, by which time head surface temperature had returned to baseline values of 35.5±0.5°C. Halothane was discontinued after completion of the surgery. After a period of physiological stabilization of roughly 20 minutes, rats were given 5 mg/kg of the ganglion blocker trimethaphan (Arfonad, Hoffman-LaRoche, Toronto, Canada) intravenously to counteract the hypertensive response during carotid clamping. This allowed for maintenance of the intraischemic blood pressure at very close to 50 mm Hg in all groups (Table 1) by controlled exsanguination through the central venous line. During the subsequent drop in blood pressure, blood was withdrawn into the heparinized syringe to further lower the blood pressure to 50 mm Hg, at which time two Moria vessel clamps were placed on the carotid arteries. Blood pressure was thereafter maintained at 50 mm Hg by withdrawal and reinfusion of the shed blood through the central venous catheter.

**Table 1. Physiological Parameters in Groups of Control and Treated Wistar Rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Nimodipine</th>
<th>MK-801</th>
<th>Nimodipine plus MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>8</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic</td>
<td>8.1±1.0</td>
<td>7.4±1.9</td>
<td>8.6±0.6</td>
<td>7.3±1.3</td>
</tr>
<tr>
<td>Postischemic</td>
<td>14.3±1.0</td>
<td>11.1±3.2</td>
<td>13.5±2.3</td>
<td>11.8±3.0</td>
</tr>
<tr>
<td>Pco2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic</td>
<td>31±5</td>
<td>30±6</td>
<td>33±4</td>
<td>34±5</td>
</tr>
<tr>
<td>Postischemic</td>
<td>37±5</td>
<td>32±3</td>
<td>35±3</td>
<td>36±1</td>
</tr>
<tr>
<td>Po2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic</td>
<td>106±21</td>
<td>123±29</td>
<td>134±30</td>
<td>104±20</td>
</tr>
<tr>
<td>Postischemic</td>
<td>133±23</td>
<td>130±14</td>
<td>142±16</td>
<td>134±24</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic</td>
<td>7.48±0.08</td>
<td>7.44±0.10</td>
<td>7.47±0.03</td>
<td>7.49±0.02</td>
</tr>
<tr>
<td>Postischemic</td>
<td>7.35±0.06</td>
<td>7.38±0.04</td>
<td>7.36±0.06</td>
<td>7.34±0.05</td>
</tr>
<tr>
<td>Blood pressure (intraischemic)*</td>
<td>48.8±1.3</td>
<td>49.1±0.7</td>
<td>49.8±0.6</td>
<td>49.1±0.5</td>
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<tr>
<td>Temperature (°C)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Intraischemic</td>
<td>34.0±0.5</td>
<td>34.0±0.5</td>
<td>34.0±0.5</td>
<td>34.0±0.5</td>
</tr>
<tr>
<td>Postischemic</td>
<td>35.5±0.5</td>
<td>35.5±0.5</td>
<td>35.5±0.5</td>
<td>35.5±0.5</td>
</tr>
</tbody>
</table>

Data are mean±SD. Preischemic values were measured 10 minutes before ischemia, and postischemic values were taken after 20 minutes of reperfusion.

*Raw data consisted of blood pressure sampled every 6 seconds during ischemic period.

**Temperatures were measured with probes inserted into temporalis muscle, estimating brain temperature minus 0.5°C in this temperature range.**

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intraperitoneal bolus injection of vehicle. Although ethanol exhibits NMDA receptor antagonism,²⁰ the effect of the ethanol vehicle was not studied here because previous work has shown that ethanol itself has an insignificant effect on ischemic brain damage.²¹,²² Treated animals received either nimodipine (0.25 µg/min x 24 hours i.v. infusion beginning 20 minutes after ischemia, n=8), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; 5 mg/kg i.v. 20 minutes after ischemia, n=12), or both regimens in combination (n=8). The tail vein catheters were doubly sutured in place and the sutures looped through the skin. In addition, their exit points from the skin were wrapped in tape. These measures, and the obtundation of the rats during the initial 24-hour postischemic period, prevented the rats from removing the catheters. All animals received single doses of diazepam (0.5 mg in 0.1 ml vehicle i.m. 20 minutes and 18–24 hours after ischemia) to reduce mortality due to postischemic seizure activity seen after such a severe insult using this model.²³

Until the rats were extubated after resumption of respiratory efforts, the temporalis muscle probes were left in place and the head temperature controlled to preischemic values. This period was longer for the two groups receiving MK-801 (approximately 1 hour) versus the other two groups (approximately ½ hour). Thereafter, all animals were left under heating lamps overnight at a distance from the lamps that produced corporeal normothermia. After a 7-day survival period, the rats were anesthetised in 2–3% halothane and intubated, a thoracotomy was performed, and transcardiac perfusion was performed using 4% formaldehyde phosphate-buffered to pH 7.35. The brains were removed 1 day after perfusion fixation, processed in graded ethanols and xylol, and embedded in paraffin. Subserial sectioning of the entire brain at 200-µm intervals was done to obtain identical sections for quantification of the cerebral cortex, hippocampus, caudate nucleus, diencephalon, brain stem, and cerebellum in all animals.

The hippocampus was assessed at five specific levels identified by their configuration in coronal sections (Figure 1). The model used produces near-complete cerebral ischemia at septal levels 1–3 (Figure 1) but temporally, blood flow from the posterior circulation results in incomplete ischemia at levels 4 and 5 (Figure 1).¹⁵ However, all control animals showed severe damage at level 5 in the ischemic penumbra. For this reason, hippocampal septotemporal levels 1–5 were assessed individually for histopathologic damage. Neuronal death was quantified blinded to the animal group. Counts of acidoophilic neurons, known from previous work to be necrotic, were obtained by direct visual examination at each of the five specific septotemporal hippocampal levels extending from areas of dense ischemia septally to areas of incomplete ischemia temporarily (Figure 1), chosen from the step sections. The cytoplasmic and nuclear outlines of the acidoophilic

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**FIGURE 1.** Effect of nimodipine, MK-801, or nimodipine plus MK-801 on hippocampal CA1 neuronal necrosis at each septotemporal level after 10.5 minutes of transient forebrain ischemia showing percent necrotic cells at septotemporal levels 1–5 and mean damage (µ) of CA1 over all levels. The percent CA1 necrosis at each septotemporal level is shown for each group (±SEM). 1) Control: 10.5 minutes of ischemia, vehicle i.v. infusion × 24 hours or single i.p. bolus injection. 2) Nimodipine: 0.25 µg/min i.v. infusion × 24 hours beginning 20 minutes after ischemia. 3) MK-801: 5 mg/kg i.v., 20 minutes after ischemia. 4) Nimodipine + MK-801: Regimens 2 and 3 combined. Note absence of a consistent effect with any treatment if only (densely ischemic) septal levels (1–3) of hippocampus are examined. Neuronal necrosis is significantly reduced by all three treatments only in the penumbra zone, where in this model the vertebrobasilar circulation supplies some blood to the temporal hippocampus,¹⁵ producing incomplete ischemia. *p<0.05, **p<0.01 compared with control.
neurons were indistinct, respectively indicating cytorhexis and karyorrhexis. The number of necrotic CA1 neurons was divided by the total number of neurons at each septotemporal level and multiplied by 100 to give the percent neuronal necrosis of each level. If virtually all CA1 neurons were necrotic in a given section, the number of normal neurons was subtracted from the total number of neurons at that particular septotemporal level to obtain the number of necrotic neurons.

In the cerebral cortex, quantified at the coronal level of the subfornical organ, the total number of acidophilic neurons in all cortical laminae was estimated by direct visual counting under the light microscope and categorized. An estimate of 10–100 necrotic neurons per section was assigned a 1, 100–1,000 neurons a 2, and >1,000 necrotic neurons per section a 3. If there were <10 necrotic neurons per section, a 0 was scored for that hemisphere. These ranked, logarithmic scores ranging from 0 to 3 (Figure 2) were summed for the two hemispheres (maximum score of 6) to obtain a cortical damage score for each animal for statistical purposes (see below).

The caudate nucleus was quantified at the coronal level of the septal nuclei at their widest point. The caudate nucleus was quantified at the coronal level of the subfornical organ. Data for left and right hemispheres are shown individually. For statistical analysis at coronal level of subfornical organ. Data for left and right hemispheres are shown individually. For statistical analysis (see Table 2), scores for the two sides were combined additively. Only the double-treated group proved statistically significant (Table 2). Drug regimens are described in Figure 1 legend.

Statistical analysis was designed to determine differences among any of the four groups and was carried out as follows. In the hippocampus, the percent CA1 necrosis at a particular septotemporal level, being a proportion, was converted using the arcsine transformation before analysis of variance and Scheffé’s test to detect specific group differences. In the cerebral cortex, the ranking scores generated for the two hemispheres in each brain were summed to give a single figure for each animal to avoid overestimating the probabilities if the hemispheres were considered independently. These neocortical scores were compared using the Bonferroni-corrected Wilcoxon rank sum test. The number of animals showing striatal necrosis and the number of animals dying (mortality) were compared using Fisher’s exact test. The resulting probabilities for cortex, hippocampus, and striatum are given in Table 2. Only the probabilities resulting from comparisons with the control group are shown, none of the probabilities resulting from comparisons among the three treatment groups being significant.

Results

Physiological parameters before, during, and after the ischemic period showed no significant differences (Table 1). However, the clinical course was different in treated and untreated animals. Rats not receiving MK-801 could be extubated earlier than those receiving the drug because of earlier resumption of respiratory efforts. Animals in the two MK-801–treated groups remained in a state of catalepsy, or wakeful immobility, from which they could not be roused for 24–36 hours after surgery. They failed to groom themselves, and lay motionless in the cage with their eyes open for the first day. On the second day after ischemia, MK-801–treated

<table>
<thead>
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<th>TABLE 2. Statistical Methods of Comparison, by Brain Region, of Results in Control and Treated Rat Groups</th>
</tr>
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<tbody>
<tr>
<td>Hippocampus</td>
</tr>
<tr>
<td>ANOVA on arcsine transformed percentage (CA1 necrosis) data, with Scheffé’s multiple comparison test to compare treated groups with controls.</td>
</tr>
<tr>
<td>Level*</td>
</tr>
<tr>
<td>Nimodipine MK-801 Both</td>
</tr>
<tr>
<td>Septotemporal level 1 0.056 0.012 0.745</td>
</tr>
<tr>
<td>Septotemporal level 2 0.124 0.018 0.698</td>
</tr>
<tr>
<td>Septotemporal level 3 0.387 0.057 0.788</td>
</tr>
<tr>
<td>Septotemporal level 4 0.233 0.023 0.006</td>
</tr>
<tr>
<td>Septotemporal level 5 0.003 0.003 &lt;0.001</td>
</tr>
<tr>
<td>Cerebral cortex</td>
</tr>
<tr>
<td>Wilcoxon rank sum test, Bonferroni-corrected probability, control group versus:</td>
</tr>
<tr>
<td>Nimodipine 0.272</td>
</tr>
<tr>
<td>MK-801 0.125</td>
</tr>
<tr>
<td>Nimodipine plus MK-801 0.046</td>
</tr>
<tr>
<td>Caudate nucleus</td>
</tr>
<tr>
<td>Fisher’s exact probability, control group versus:</td>
</tr>
<tr>
<td>Nimodipine 0.121</td>
</tr>
<tr>
<td>MK-801 0.059</td>
</tr>
<tr>
<td>Nimodipine plus MK-801 0.018</td>
</tr>
<tr>
<td>*See Figure 1.</td>
</tr>
</tbody>
</table>

*See Figure 1.
plus MK-801 on the frequency of neuronal necrosis in the caudate nucleus after 10.5 minutes of transient forebrain ischemia. Drug regimens are described in Figure 1 legend. In the caudate, damage was present in five of eight untreated control animals, one of six nimodipine-treated animals, and one of eight MK-801-treated animals, but no neuronal necrosis was seen in the caudate nuclei of any animal receiving combined therapy. Only reduction due to combined therapy is statistically significant (*p<0.05; Table 2).

FIGURE 3. Effect of nimodipine, MK-801, or nimodipine plus MK-801 on the frequency of neuronal necrosis in the caudate nucleus after 10.5 minutes of transient forebrain ischemia. Drug regimens are described in Figure 1 legend. In the caudate, damage was present in five of eight untreated control animals, one of six nimodipine-treated animals, and one of eight MK-801-treated animals, but no neuronal necrosis was seen in the caudate nuclei of any animal receiving combined therapy. Only reduction due to combined therapy is statistically significant (*p<0.05; Table 2).

Rats showed ataxia on walking. By the fourth posts ischemic day, they were indistinguishable from untreated ischemic controls and nimodipine-treated rats. The neurobehavioral effects of dizocilpine and their time course thus resembled those seen in naive rats given dizocilpine, and may be accounted for by NMDA receptor blockade. Mortality, mostly seizure related, was 50% in the untreated animals, and 25%, 33%, and 12.5% in the rats receiving nimodipine, MK-801, and both therapies, respectively. None of these differences in mortality were statistically significant (Fisher's exact test).

In the hippocampus, results revealed little protection in the septal, densely ischemic portion of the hippocampus (Figure 1). Only the MK-801–treated group showed inconsistent protection at some of the three most septal levels, with probabilities in the range of 0.012 < p < 0.057 (Table 2). Temporally, however, where ischemia is incomplete in this model, the reductions in neuronal necrosis were highly significant (see Table 2).

Analysis of cortical and striatal damage showed significant differences only between the control group and those receiving combined therapy. In the neocortex, neuronal necrosis was least severe in the double-treated group (Figure 2). In the caudate nucleus (Figure 3), combined therapy eliminated damage entirely (Figure 4) in all animals (p = 0.018). The reduction in striatal necrosis seen with MK-801 alone approached significance (p = 0.059).

In the brain stem, bilateral infarction of the pars reticulata of the substantia nigra was seen in five of eight controls, two of six nimodipine-treated rats, one of eight MK-801–treated rats, and two of eight dual-treated rats. None of these differences were significant.

Discussion

The present study suggests that in transient forebrain ischemia, combined treatment with nimodipine and MK-801 reduces ischemic neuronal death in several major brain regions. Because any effect of MK-801 alone is germane to the present findings, and because of controversy over the effectiveness of MK-801 alone in global ischemia, this topic will first be discussed.

MK-801 blocks ionic fluxes, including inward calcium fluxes, through channels gated by the NMDA receptor. This blockade is stronger in the presence of agonist, when MK-801 can enter the ion channel in its open conformation. This suggests MK-801 might bind selectively to ischemic tissue, where glutamate or other excitatory compounds are released. Temperature may be a factor influencing channel conformation and opening.

Initial experiments with MK-801 in global ischemia appeared highly promising, in that a robust reduction in ischemic necrosis was reported, whether the drug was given before or after ischemia, with some effect reported even after administration 24 hours after ischemia. In experiments controlling body but not brain temperature, our laboratory was unable to replicate a neuroprotective effect at postischemic intervals of 24 hours or even 2 hours, but a neuroprotective effect was seen if the drug was given 20 minutes after restoration of blood flow. Experiments in two laboratories subsequently showed that when the temperature fall induced by MK-801 was obviated, no neuroprotection was seen; this finding suggests that the operant mechanism of protection was merely drug-induced hypothermia. Small differences in intrasubicemic and postischemic (<30-minute) brain temperature have been shown to profoundly influence the degree of subsequent ischemic necrosis.

Because our initial study with MK-801 showed a degree of neuroprotection intermediate between the robust neuroprotection seen without temperature control and the completely negative findings with temperature control, we carried out another study with MK-801, this time with strict control of head temperature as well as body temperature. Neurobehavioral testing was done to gain a functional assessment of efficacy in addition to histology. The results showed that MK-801 did not reduce neuronal necrosis in the septal, densely ischemic CA1 pyramidal cells to a degree reaching statistical significance. However, sections at coronal levels in the temporal hippocampus, incompletely ischemic due to continuous vertebrobasilar supply, did show significant protection. This was enough to significantly reduce overall necrosis at all levels of the hippocampus. Also, neocortical necrosis was eliminated by MK-801. The degree of histological protection was sufficient to be accompanied by improved performance on tasks of learning and memory carried out before histological analysis.

Based on the results discussed above, it is possible to reconcile apparently discrepant findings in the literature concerning the effect of MK-801 in global ischemia. It is clear that in temperature-controlled studies obviating hypothermia, MK-801 gives no significant protective effect in global ischemia. However, in rodent experiments examining serially sectioned rat brains, protection against ischemic necrosis is seen in the temporal...
FIGURE 4. Photomicrograph of caudate nucleus demonstrating improvement in neuronal necrosis by double therapy, as seen in all animals of untreated and double-treated groups. Caudate nucleus of an untreated control animal (left panel) shows cytorrhesis of most neurons in the field, leaving only glia, whereas all animals receiving combination therapy with nimodipine and MK-801 showed total neuronal preservation (right panel) throughout entire caudate bilaterally. Stain, 2% phloxine and 0.1% cresyl violet. Bar, 100 μm.

hippocampus, in the hippocampus considered as a whole (along its entire septotemporal axis), and in the neocortex, even with rigid temperature control. That MK-801 may have a small residual benefit even without hypothermia is supported by the findings of significant neuroprotection in multiple brain regions using temperature-controlled global hypobaric-ischemic injury in infant rats, where ambient, head, and body temperatures do not fall during or after the insult, and in hypoglycemia where brain perfusion is normal and temperature, even if measured to 0.1°C, does not fall significantly.

Calcium channel blockers, used alone, appear to show only a weak protective effect in transient global ischemia, similar to that of NMDA antagonists. Blockers of the L-type voltage-sensitive calcium channel in ischemia have recently been reviewed. In global cerebral ischemia, studies have yielded both positive and negative results. Similar to the pattern of results emerging with MK-801, investigations using nimodipine are more decidedly positive in focal ischemia than in global ischemia. Also, both animal and human studies finding significant benefit have tended to use continuous intravenous nimodipine infusion, as we did in the present study. Early intervention after ischemia also seems to increase the chance of an effect being shown with postischemic nimodipine in both animals and humans.

The weak neuroprotective effect in global ischemia seen individually with either NMDA antagonists or calcium channel blockers did not reach statistical significance in all brain regions in the present study. If the two groups treated with a single drug had been the only treatment groups done, they would have been significantly different on comparison with the untreated controls. However, such an approach is not statistically valid if additional groups, in this case the double-treated group, have actually been done in the same study. We may conclude from the present investigation that the weak neuroprotective effects of either MK-801 or nimodipine (see above) have each been augmented by addition of the other drug, giving rise to a significant protective effect against damage in several brain regions. A similar effect, using the identical combination of drugs, has recently been shown in focal cerebral ischemia.

Because intraischemic temporalis muscle temperatures were controlled to identical readings during and after the ischemia (Table 1) and head temperatures had normalized to preischemic levels (Table 1) by 20 minutes after the ischemia, when all drugs were administered, intraischemic hypothermia interacting with MK-801 (or nimodipine) cannot be invoked to explain the neuroprotection. Neither can postischemic hypothermia explain the neuroprotection seen, because postischemic temperature was monitored (longest in the MK-801-treated groups) and the animals were left under heating lamps after the ischemia. We conclude that the most likely mechanism of action is through an effect of the drugs on central nervous system tissue involving blockade of calcium influx.

Because calcium entry could occur by mechanisms unblocked by even the two drugs used in the present
study, the addition of still other therapeutic strategies aimed at mitigating depolarization or calcium influx might provide a still greater neuroprotective effect. Thus, although the results of the present investigation cannot be ascribed to hypothermia induced by MK-801, they do not weigh against an additive beneficial effect of hypothermia on MK-801, which has in fact been shown.19

If calcium entry is indeed critical in the pathogenesis of ischemic cell death, then all sources of significant elevation in cytosolic calcium should be considered. The present combination of drugs still allows unhindered neuronal calcium influx through dihydropyridine-sensitive, N-type voltage-sensitive calcium channels linked to neurotransmitter release, and through nonspecific cation channels.40 42 Intracellular redistribution of calcium could also play a role in calcium-related events and would also be unaffected by the channel blockers used in the present study. Release could theoretically occur from the mitochondria or from the endoplasmic reticulum, but only release from the latter site seems large enough to raise cytosolic calcium.43

Lastly, ischemia could directly activate "metabotropic" receptors.44 Mobilization of intracellular calcium is a prominent feature of activation of these receptors45 and would also bypass extracellular calcium entry as a pathogenetic mechanism. Such metabotropic cellular activation44 45 and toxicity46 is preferentially activated by non-NMDA over NMDA receptors and would be unmitigated by nimodipine or MK-801.

Thus, cytosolic calcium levels could be raised by mechanisms unaffected by the channel blockers used in the present study. Blockade of additional types of calcium channels and of non-NMDA excitatory receptors might give an even greater degree of neuroprotection against ischemia. In particular, the combination of NMDA and non-NMDA antagonists might prove more potent against ischemic neuronal necrosis, given recent findings in cell culture47 and in vivo.48 Combination therapy, using individual agents each acting on a different mechanism contributing to cell necrosis, would seem a rational approach for future therapy in global cerebral ischemia.

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