Combined Treatment With MK-801 and Nicardipine Reduces Global Ischemic Damage in the Gerbil

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Background and Purpose: Excessive activation of the N-methyl-D-aspartate receptor by glutamate produces an influx of Ca\(^{2+}\), which in turn is thought to lead to ischemic cell death. In this study we evaluated the combined treatment of the N-methyl-D-aspartate antagonist dizocilpine (MK-801) and the dihydropyridine Ca\(^{2+}\) channel blocker nicardipine for the reduction of hippocampal CA1 neuronal loss.

Methods: Global ischemia was induced by bilateral carotid artery occlusion in the gerbil. Body temperature was maintained between 36.5°C and 37.5°C during surgery. MK-801 (5.0 mg/kg) was injected 15 minutes after occlusion whereas nicardipine was given by injection and via a micro-osmotic pump (1.0 mg/kg/day) for 3 days.

Results: Postischemic treatment with MK-801 reduced CA1 cell loss by 27.0%, whereas nicardipine reduced CA1 cell loss by 13.3%. Combined postischemic treatment with these drugs yielded an additive, protective effect (44.3% reduction of CA1 loss) that did not appear to result from postischemic hypothermia as assessed by skull and rectal temperature recordings.

Conclusions: Our results demonstrate that MK-801 plus nicardipine significantly attenuates CA1 cell death after forebrain ischemia in the gerbil. Excitatory amino acid antagonists in combination with Ca\(^{2+}\) channel antagonists may be an effective therapy in patients exposed to global ischemic insult. (Stroke 1992;23:82-86)

The CA1 sector of the hippocampus is particularly sensitive to ischemic insult.\(^1\)-\(^5\) The cell death occurring in the CA1 sector has been described as "delayed neuronal death"\(^6\)-\(^7\) because its time course appears to be considerably longer than that typical of ischemic neuronal death in other brain regions. The pathophysiological precedents of ischemic neuronal death have been attributed to several causes; however, recent research has focused on the excitatory amino acid, glutamate\(^8\) as well as Ca\(^{2+}\)\(^9\)-\(^11\)

During an ischemic episode there is a large increase in extracellular levels of glutamate because of enhanced release and reuptake failure.\(^12\) The increased concentration of glutamate causes prolonged activation of the N-methyl-D-aspartate (NMDA) receptor\(^8\) which in turn leads to an excessive influx of Na\(^{+}\), Ca\(^{2+}\), and Cl\(^{-}\) through the NMDA-associated ion channel. In addition, glutamate-induced depolarization activates voltage-gated Ca\(^{2+}\) channels.\(^10\) Finally, Ca\(^{2+}\) may enter the cell via a malfunction in the Na\(^{+}\)-Ca\(^{2+}\) exchanger, or possibly through a nonspecific membrane leak.\(^10\),\(^11\) The increase in intracellular Ca\(^{2+}\) activates intracellular lipases and proteases and a cascade of other biochemical processes that are incompatible with neuronal viability.\(^10\),\(^11\)

Competitive (CGS-19755) and noncompetitive (e.g., MK-801) NMDA receptor antagonists have been reported to attenuate global ischemic damage\(^13\)-\(^17\); however, these findings are being reassessed in view of more recent data showing that MK-801′s protective actions may be caused by drug-induced hypothermia.\(^18\),\(^19\)

Nicardipine, a Ca\(^{2+}\) channel blocker of the dihydropyridine type, reduces ischemic cell damage\(^20\) in the gerbil, whereas in the rat, its protective effects have been greatest on functional, rather than histological, outcome.\(^21\)-\(^23\) Recently, nicardipine has been reported to improve outcome in stroke patients.\(^24\) Other Ca\(^{2+}\) channel antagonists such as nimodipine and flunarizine have yielded mixed results.\(^25\)-\(^28\)

Individual treatment with MK-801 or nicardipine appears to offer some degree of protection against global ischemic insult; however, a combination of...
these drugs has not been evaluated. The following experiment examines the protective effects of MK-801 and nicardipine alone and in combination in reducing CA1 ischemic damage. A combined treatment of MK-801 and nicardipine would be expected to be more potent than either drug alone since the combined drug treatment should limit Ca\textsuperscript{2+} entry through both the NMDA-associated ion channel and the voltage-operated channels.

**Materials and Methods**

Forty-eight female Mongolian gerbils (High Oak Ranch Ltd., Goodwood, Canada) weighing 50–70 g were used in this experiment. Animals were anesthetized with 2% halothane mixed with oxygen (30%) and nitrous oxide (70%) via a mask-equipped Fluovac Halothane Scavenger system (Stoelting Co., Chicago, Ill.). Surgery was performed under a fiber-optic cold light source at a room temperature of 22°C.

The carotid arteries were isolated, separated from the vagus nerve, and then clamped with microarterial clamps (Fine Science Tools, Vancouver, Canada) for 5 minutes. The clamps were then removed, and reflow was verified. The incision was closed with sutures, and the animals were removed from anesthesia and placed under a 60-W laboratory lamp (38.0 cm from the cage floor) to recover for approximately 1 hour. Throughout surgery all animals were anesthetized with 2% halothane mixed with oxygen (30%) and nitrous oxide (70%) via a mask-equipped Fluovac Halothane Scavenger system (Stoelting Co., Chicago, Ill.). Surgery was performed under a fiber-optic cold light source at a room temperature of 22°C.

Before surgery the animals were divided into four groups of 12 gerbils each. The MK gerbils received an intraperitoneal injection of MK-801 (5.0 mg/kg) dissolved in saline, 15 minutes after carotid artery occlusion. The N animals received an intraperitoneal injection of nicardipine (0.5 mg/kg) dissolved in distilled water, 15 minutes after carotid artery occlusion. The N animals also received a slow, continuous administration of nicardipine (1 mg/kg/day for 3 days) by means of a micro-osmotic pump (Alzet model 1003D, Alza Corp., Palo Alto, Calif.) implanted subcutaneously in the animal’s back. All pumps were primed for 4 hours at 37°C in physiologic saline before implantation to ensure a steady release rate after insertion. The micro-osmotic pump was removed 5 days after surgery under halothane anesthesia. The NMK group represented a combination of both MK and N treatments. All animals received an injection of both MK-801 (5.0 mg/kg i.p.) and nicardipine (0.5 mg/kg i.p.) 15 minutes after the carotid artery occlusion. They also were implanted with the micro-osmotic pump containing the same dose of nicardipine as the N group. The S group served as a control for surgical, injection, pump implantation, and pump removal procedures. Animals in this group were occluded and received saline instead of MK-801 or nicardipine.

Postischemic rectal temperatures and skull temperatures were recorded from 4 gerbils of the NMK group. Skull temperature was measured using a 30-gauge stainless steel thermocouple probe (Omega Engineering, Stamford, Conn.) inserted subcutaneously over the midline of the skull while the gerbils were under halothane anesthesia. The sedative effects of MK-801 allowed the probes to be left in place for the first hour of reperfusion.

Ten days after occlusion, animals were given an overdose of sodium pentobarbital (50 mg/kg i.p.), and their brains were perfusion fixed using physiological saline (30 ml) followed by 10% phosphate-buffered formalin (30 ml). The brains were then removed and stored in 10% formalin for at least 2 days. A day before sectioning, brains were transferred to a 20% sucrose/formalin solution. The tissue was frozen and sliced at 10 μm (a few brains were cut at 20 and 40 μm). The sections were then stained with cresyl violet.

The extent of CA1 damage was assessed by rating the percentage of normal neurons according to a 5-point scale where 90–100% was scored as 4, 60–89% was 3, 30–59% was 2, 6–29% was 1, and less than 5% was 0. Sectors corresponding to the medial, middle, and lateral aspects of CA1 were rated from the histological sections of the gerbils (authors' unpublished observations).

**Results**

All 48 gerbils subjected to ischemia recovered well, and there was no incidence of seizures. The brain from one animal in the MK-801 group was damaged during processing and had to be discarded. Postischemic skull and rectal temperatures (Table 1) of the gerbils given the combined treatment of nicardipine and MK-801 were within the normal range.

Figure 1 shows the CA1 rating scores for each experimental group. Two-tailed Mann-Whitney U tests revealed that the combination of MK-801 and nicardipine significantly attenuated CA1 cell loss (U_{12,12}=17.5, p<0.01) as did MK-801 by itself.
FIGURE 2. Representative photomicrographs illustrating each of the five CA1 rating scores, from the top: 4, 90–100% of cells are intact; 3, 60–89% of cells are intact; 2, 30–59% of cells are intact; 1, 6–29% of cells are intact; and 0, less than 5% of cells are intact. Cresyl violet stain. Original magnification, ×160.

Discussion

The present results show that a marked reduction of CA1 cell damage was achieved with postischemic administration of MK-801 plus nicardipine. By itself, MK-801 was also effective in reducing CA1 damage; however, the protective effects were less consistent than with the combined drug treatment. The most likely explanation of these findings is that nicardipine and MK-801 in combination are able to reduce ischemic injury by blocking the NMDA-associated ion channel and voltage-operated Ca\textsuperscript{2+} channels. Blockade of these channels should reduce two routes of intracellular Ca\textsuperscript{2+} entry that have been implicated in ischemic cell death.\textsuperscript{10,11} In support of this interpretation are results showing that MK-801 and the Ca\textsuperscript{2+} channel blocker nimodipine given during middle cerebral artery occlusion in the cat reduced cortical damage and facilitated postischemic recovery of electroencephalographic activity.\textsuperscript{30} In this study, fluorometric techniques for measuring intracellular Ca\textsuperscript{2+} entry revealed that while MK-801 attenuated postischemic influx of Ca\textsuperscript{2+} into cortical neurons, MK-801

(U\textsubscript{11,20}=30, p<0.05). Nicardipine alone produced little benefit; the animals on average were nearly as damaged as nontreated animals subjected to ischemia.

Photomicrographs illustrating examples of each of the damage ratings (0–4) are shown in Figure 2. CA1 cell loss was severe in the S group with nine of 12 animals having damage ratings less than 6. The results were more variable in the N group; seven gerbils had severe damage and were rated at 6 or less while the remaining five animals were either moderately (n=3) or well protected (n=2). A bimodal distribution of damage was observed in the MK-801 group with six gerbils showing severe damage and five showing moderate to near total protection. The combined treatment group (NMK) yielded the best results: six animals had rating scores of 20 or better, three showed evidence of moderate protection and only three gerbils had ratings of less than 8.

FIGURE 1. CA1 rating scores for each group: S (saline), N (nicardipine), MK (MK-801), and NMK (nicardipine plus MK-801). Three sectors (medial, middle, and lateral) of CA1 from both hemispheres were rated on a 5-point scale (see “Materials and Methods”) to yield an overall rating. A score of 24 (6×4) represents an intact CA1 while a score near 0 represents severe neuronal loss. Filled circles indicate that two animals had the same rating. Horizontal lines illustrate the mean rating scores for each group. NMK vs. S, p<0.01; MK vs. S, p<0.05, Mann-Whitney U test.
plus nimodipine was even more effective, normalizing levels of Ca\(^{2+}\) during occlusion and the reperfusion period. It is likely that the combined drug treatment in our study had similar effects on Ca\(^{2+}\) influx into CA1 neurons.

It is important to exclude the possible confounding effects of hypothermia from any drug experiment since hypothermia during ischemia\(^{18,19,31-33}\) or the early postischemic phase\(^{34,35}\) can result in significant neuronal protection, which then could be erroneously attributed to a particular drug action (e.g., blockade of NMDA receptors). In the present study hypothermic effects were ruled out because temperature was maintained at normal levels during surgery and the MK-801 was administered postschemically in conscious, nonanesthetized animals. MK-801 does not produce hypothermia in nonanesthetized animals (authors’ unpublished observations). Also, hypothermia cannot explain the robust protective effects of MK-801 given in combination with nicardipine. These animals (Table 1) were not hypothermic except perhaps at the end of surgery (approximately 10–12 minutes after occlusion), when rectal temperature dropped transiently to 35.9°C. This drop coincided with the implantation of the micro-osmotic pump, when the animals were removed from the heating blanket and rotated onto their abdomens. Shaving the fur and opening the skin to implant the pump also likely contributed to this small temperature drop. In any case, rectal temperature had returned to normal levels within 30 minutes of occlusion. In the two studies\(^{34,35}\) that have systematically examined the protective effects of postischemic hypothermia, both the degree (30–34°C) and duration (2–3 hours) of postischemic hypothermia were far greater than what our animals experienced. More relevant are the postischemic skull temperature data, which in the same NMK animals fell only slightly at the end of surgery (36.3°C) and 10 minutes later were at a normal level (37.3°C), rising above 38°C during the next 50 minutes.

While Alps et al\(^{20,23}\) found cellular protection with nicardipine alone, it was not found in the present study. The reasons for this are not clear; however, the timing of drug administration is different in each study. All drug treatments in the present study were postischemic, whereas Alps et al\(^{20,23}\) delivered nicardipine both before and after occlusion. A more important variable may be temperature during ischemia. In previous studies that have reported beneficial effects of nicardipine\(^{20,23}\) body temperature was not controlled during surgery and thus may have contributed to the neuroprotective effects observed.\(^{36}\)

The incomplete cellular protection observed in this study suggest that other factors are playing a role in ischemic brain damage. Recently, a potent and selective antagonist of the quisqualate receptor 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) has been reported to protect against CA1 neuronal loss in the Mongolian gerbil after 5 minutes of carotid artery occlusion, even when administered 2 hours after ischemia.\(^{37}\) Thus, non-NMDA receptors would appear to have a more important role in ischemic brain damage than previously thought. Furthermore, other dihydropyridine Ca\(^{2+}\) channel blockers such as nimodipine seem to be more effective than nicardipine in both global and focal ischemic damage.\(^{25,26,38}\) Similarly, a phenylalkylamine Ca\(^{2+}\) antagonist, (s)-emopamil, has been reported to be a more effective treatment for cerebral ischemic brain damage than certain dihydropyridine-type antagonists.\(^{39}\) This drug, which readily penetrates the blood–brain barrier, selectively antagonizes brain artery Ca\(^{2+}\) channels and is a potent serotonin-receptor antagonist.\(^{39}\) Since serotonin is involved in vasoconstriction and platelet aggregation,\(^{39}\) (s)-emopamil may be a particularly effective therapeutic agent. In conclusion, it can be stated that drug treatments involving either NMDA or non-NMDA antagonists in combination with Ca\(^{2+}\) channel blockers may be a promising avenue for future treatment of cerebral ischemic injury.

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