Competitive N-Methyl-d-Aspartate Receptor Blockade Reduces Brain Injury Following Transient Focal Ischemia in Cats

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Background and Purpose We tested the hypothesis that administration of the competitive N-methyl-d-aspartate (NMDA) receptor antagonist NPC 17742 (2R,4R,5S-[2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid]) during transient focal ischemia affects early postischemic brain injury.

Methods Halothane-anesthetized cats underwent 1 hour of left middle cerebral artery occlusion plus 4 hours of reperfusion. Control cats received saline (n=7). Experimental cats were treated with NPC 17742 at a dose of 5 mg/kg IV from 45 minutes of ischemia to 15 minutes of reperfusion and 2.5 mg/kg per hour for 4 hours of reperfusion (NPC-5; n=7) or 50 mg/kg from 45 minutes of ischemia to 15 minutes of reperfusion and 25 mg/kg per hour for 4 hours of reperfusion (NPC-50; n=5).

Results Microsphere-determined blood flow to the ipsilateral inferior temporal cortex and caudate nucleus decreased to the same extent during ischemia and recovered to the same extent during reperfusion in the three groups. Triphenyltetrazolium-determined injury volume of ipsilateral cerebral hemisphere; mean±SE) and caudate nucleus (saline, 72±6%; NPC-5, 37±10%; NPC-50, 26±4%) was less in cats treated with both doses of drug compared with cats treated with saline. Recovery of somatosensory evoked potential amplitude was incomplete and similar in all groups (saline, 36±14%; NPC-5, 58±8%; NPC-50, 51±13% of baseline).

Conclusions These data indicate that activation of NMDA receptors plays an important role in the mechanism of acute injury in both cortex and caudate after 1 hour of transient focal ischemia in the cat. Because NPC 17742 afforded protection when administered at the end of ischemia and during reperfusion, NMDA receptor activation during reperfusion may contribute to the progression of injury in ischemic border regions.

Key Words • cerebral blood flow • evoked potentials, somatosensory • excitatory amino acids • middle cerebral artery occlusion

Excitatory amino acids have been implicated as important mediators of neuronal injury during focal ischemia.1,2 Once produced, excitatory amino acids are believed to produce neurotoxicity by a mechanism that involves stimulation of both N-methyl-d-aspartate (NMDA) and non-NMDA excitatory amino acid receptors. MK-801 (dizocilpine) is a noncompetitive NMDA receptor antagonist that has been demonstrated to decrease brain injury after permanent3,4 and transient5 focal ischemia in cats. However, doses of MK-801 that afford cerebral protection also produce significant sedation6 and reduction in cerebral metabolism in the absence of ischemia.7 In addition, several of the noncompetitive NMDA receptor antagonists, including MK-801, can cause alterations in neuronal morphology and induction of heat-shock proteins.8,9 More recently several specific competitive NMDA receptor antagonists have been developed that cross the blood-brain barrier. Competitive NMDA receptor antagonists have therapeutic effects at doses much lower than those required to impair motor performance.10 Treatment with other competitive NMDA receptor antagonists has been demonstrated to reduce injury that follows permanent focal ischemia.11,12 However, efficacy of competitive NMDA antagonists administered at reperfusion after transient focal ischemia is unclear. The goal of the present study was to test the hypothesis that a competitive NMDA receptor antagonist decreases the volume of injury associated with 1 hour of transient focal ischemia when the drug is administered at two different doses 15 minutes before reperfusion. Intravenous infusion was started 15 minutes before reperfusion to permit delivery of the drug to ischemic tissue with low blood flow. We also measured cerebral blood flow (CBF) to determine if the mechanism of protection was related to a redistribution of cerebral perfusion during either ischemia or reperfusion.

Methods Focal ischemia was produced in 19 female cats weighing 3.0±0.1 kg. After anesthesia was induced with halothane in oxygen, cats were orally intubated and mechanically ventilated to maintain Paco2 at approximately 35 to 40 mm Hg. Anesthe-
sia was maintained with halothane (0.5% to 1.5%) in oxygen-enriched air (FiO₂, 0.35 to 0.40). Anesthetic concentration was not altered during ischemia and reperfusion. Pancuronium bromide (0.2 mg/kg IV) was administered as a single dose, for muscle relaxation to prevent movement during electrocatheter and muscle artifact during evoked potential monitoring.

Both femoral veins were catheterized for infusion of lactated Ringer’s solution and drugs. Catheters were placed in the descending aorta through a femoral artery for blood pressure measurement, arterial blood gas sampling, and for withdrawal of reference blood samples during injection of radiolabeled microspheres. After left thoracotomy, a catheter was inserted into the left atrium for injection of radiolabeled microspheres. The cat was turned prone, and its head was positioned in a stereotactic frame approximately 4 cm higher than its heart. A thermistor placed in the right temporal epidermal space was used to estimate brain temperature. Epidermal temperature was maintained at 38.0±0.5°C using a warmed water blanket and a heating lamp. The left middle cerebral artery (MCA) was exposed by a transorbital approach using microsurgical techniques. To produce focal ischemia, the left MCA was occluded near its origin from the intracranial carotid artery using a microvascular clip for 1 hour. At 1 hour of ischemia, the microvascular clip on the MCA was removed. Reperfusion lasted 4 hours.

Arterial blood pressure was continuously monitored. Arterial pH, PaCO₂, and PaO₂ were measured with a self-calibrating Radiometer electrode system (ABL3). Hemoglobin and arterial oxygen content were measured with a hemoximeter (Radiometer, model OSM3). Blood glucose was measured with a glucose analyzer (model 2300A, Yellow Springs Instruments). A multichannel signal averager (model CA-1000, Nicolet Biomedical Instruments) was used to measure the somatosensory evoked potential (SEP) with foreleg stimulation as previously described.13-14 The amplitude to the peak of the first major negative wave was measured from the peak of the preceding positive wave.

Regional CBF was measured with radiolabeled microspheres (16±0.5 μm in diameter; Du Pont-NEN Products) using the reference withdrawal method.15 Six radioactive isotopes (¹⁵⁰Gd, ¹ⁱ⁰In, ¹¹⁵Sn, ¹³³Ru, ⁸⁹Nb, ⁴⁸Sc) were injected in random sequence in each animal. Approximately 1.5×10⁴ microspheres were injected into the left atrium over a 20-second period, followed by a 5-mL saline flush. The reference blood sample was withdrawn from the aorta at 1.5 mL/min beginning 30 seconds before the injection and continuing for 90 seconds after the saline flush.

After obtaining baseline measurements, cats were assigned to one of three groups. Each group was exposed to 60 minutes of left MCA occlusion and 4 hours of reperfusion. In each group treatment with either saline (saline) or drug was begun at 45 minutes of MCA occlusion. Each group received 10 mL of drug or 0.9% NaCl over 30 minutes (between 45 minutes of MCA occlusion and 15 minutes of reperfusion) by continuous intravenous infusion. In the control group, after the initial saline infusion, additional saline was infused at a rate of 4 mL/h. In the second group (NPC-5), 5 mg/kg NPC 17742 was infused (total volume, 10 mL) over the first 30 minutes, as described above, followed by 2.5 mg/kg per hour (volume, 4 mL/h) for the remainder of reperfusion. In the third group (NPC-50), 50 mg/kg NPC 17742 was infused (total volume, 10 mL) over the first 30 minutes, as described above, followed by 25 mg/kg per hour (volume, 4 mL/h) for the remainder of reperfusion. Cats that did not achieve at least 75% reduction in SEP amplitude with MCA occlusion were immediately excluded from the protocol (before randomization to a particular treatment group). All variables were measured at baseline, at 30 and 60 minutes of left MCA occlusion, and at 1, 2, and 4 hours of reperfusion.

At 4 hours of reperfusion, cats in all groups were killed with intravenous potassium chloride. Brains were removed and cut immediately into 12 uniform coronal sections 3 mm thick to estimate brain injury with the 2,3,5-triphenyltetrazolium chloride (TTC) Sigma Chemical Co) technique previously described in our laboratory.15-16 After injury volume was estimated, the slices of brain were placed in 10% buffered formalin for 1 to 2 days. Ipsilateral and contralateral temporal and parietal lobes of the middle four slices were subsequently sectioned into three cortical gray matter regions (inferior temporal, lateral temporal-parietal, and superior parietal) for regional CBF determinations. Brain was then sectioned into ipsilateral and contralateral caudate nucleus, brain stem, and cerebellum. The arterial microsphere reference samples and weighed tissue specimens were counted in a multichannel autogamma scintillation spectrometer (Minaxi model 5530, Packard Instruments). The overlap of activity among isotopes was corrected by differential spectroscopy, and blood flow was calculated by the reference sample technique.15

An additional 3 cats were treated with NPC 17742 at a dose of 50 mg/kg administered intravenously over 30 minutes followed by 25 mg/kg per hour to determine the effects of NPC 17742 on CBF, cerebral oxygen consumption (CMRO₂), and SEP amplitude without intervening ischemia. These cats were instrumented similarly to those exposed to transient focal ischemia except no eye surgery was done and a sagittal sinus catheter was placed to obtain cerebral venous blood for calculation of CMRO₂. In these cats all variables were recorded at baseline and at 5 minutes, 30 minutes, 2 hours, 4 hours, and 6 hours of NPC 17742 infusion.

Values are expressed as mean±SE. Statistical comparison to assess changes in measured physiological variables within groups was performed by repeated-measures ANOVA. The effect of experimental manipulation on blood flow within each group was determined with paired Student’s t test with Bonferroni correction. Differences among groups in injury volume were determined by ANOVA and t test with Bonferroni correction. Statistical differences were considered significant at P<.05.

Results

There were no physiologically significant differences among the three experimental groups in baseline values of any physiological variables. Average mean arterial blood pressure was maintained between 85 and 120 mm Hg, and hemoglobin was maintained above 10 g/dL in all groups and at all times. Arterial glucose concentration was higher in saline-treated cats at 1 hour (saline, 138±6; NPC-5, 100±7; NPC-50, 96±11 mg/dL) and 2 hours (saline, 142±12; NPC-5, 102±10; NPC-50, 98±10 mg/dL) of reperfusion but not during ischemia or at 4 hours of reperfusion. Brain temperature was controlled at approximately 38°C in all groups.

Left MCA occlusion produced a similar degree of ischemia in all ipsilateral cortical brain regions (Table 1). Likewise, blood flow to the ipsilateral caudate nucleus was similarly reduced in all groups during MCA occlusion. In both groups treated with NPC 17742, blood flow to the caudate nucleus remained at baseline values for the first 2 hours of reperfusion, whereas it remained below baseline values throughout reperfusion in saline-treated cats. Left MCA occlusion did not affect blood flow to contralateral brain regions. There were no differences in blood flow among groups at any point within any region. This baseline amplitude (saline, 76±12 μV; NPC-5, 58±9 μV; NPC-50, 94±18 μV) and latency (saline, 12.1±0.2 milliseconds; NPC-5, 12.7±0.2 milliseconds; NPC-50, 12.1±0.2 milliseconds) of the primary cortical SEP were not different among groups. Ipsilateral SEP amplitudes

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were suppressed to the same extent during left MCA occlusion and showed similar recovery in all groups (Table 2). However, SEP amplitudes over the contralateral somatosensory cortex showed no change during 1 hour of left MCA occlusion followed by 4 hours of reperfusion in the three groups. In addition, all cats had normal latency of the wave measured over the second cervical vertebra throughout the protocol.

Ipsilateral cerebral hemispheric injury volume was significantly greater in the saline group compared with the NPC-5 and NPC-50 groups when expressed in terms of absolute units (saline, 2215±733 mm$^3$; NPC-5, 344±118 mm$^3$; NPC-50, 505±216 mm$^3$) or as a percentage of total ipsilateral hemispheric volume (Fig 1). In addition, injury volume of the caudate nucleus in saline-treated cats (209±16 mm$^3$) was greater than that in cats treated with NPC-5 (102±32 mm$^3$) or NPC-50 (71±13 mm$^3$). When expressed as a percentage of total ipsilateral caudate volume, injury volume in the saline group was also greater than injury volume in the NPC-5 and

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L-MCAO indicates left middle cerebral artery occlusion; Saline, cats treated with 10 mL 0.9% NaCl between 45 minutes of L-MCAO and 15 minutes of reperfusion followed by 4 mL/h for the remainder of reperfusion; NPC-5, cats treated with 5 mg/kg NPC 17742 in 10 mL saline between 45 minutes of L-MCAO and 45 minutes of reperfusion followed by 2.5 mg/kg per hour at a rate of 4 mL/h for the remainder of reperfusion; and NPC-50, cats treated with 50 mg/kg NPC 17742 in 10 mL saline between 45 minutes of L-MCAO and 15 minutes of reperfusion followed by 25 mg/kg per hour at a rate of 4 mL/h for the remainder of reperfusion. Values are mean±SE. *P<.05 vs baseline. No differences between groups at any point within any region.
NPC-50 groups (Fig 1). When injury volume was analyzed separately for each of 12 coronal sections, injury was reduced in the ipsilateral hemisphere and ipsilateral caudate nucleus in both groups treated with NPC (two-way ANOVA), but there was no difference between NPC groups (Fig 2).

In nonischemic cats (n=3) there was no effect of high-dose NPC 17742 on CMRO2 (in mL/min per 100 g: baseline, 4.1±0.8; 5 minutes, 4.6±0.7; 30 minutes, 5.0±1.4; 2 hours, 4.3±0.1; 4 hours, 5.1±0.4; 6 hours, 5.0±0.7). CBF decreased from 184±31 to 114±19 mL/min per 100 g after 6 hours of NPC 17742 administration. Amplitude of SEP decreased to 58±6% of baseline amplitude 5 minutes after administration of NPC 17742 and was 70±4% of baseline after 6 hours of NPC 17742 administration.

**Discussion**

This study demonstrated that intravenous administration of NPC 17742 beginning at 45 minutes of ischemia and continuing throughout 4 hours of reperfusion substantially reduced brain injury volume during transient focal ischemia. Attenuation of injury volume in the cerebral hemisphere and caudate nucleus was not due to an alteration in regional CBF during MCA occlusion or reperfusion. Despite attenuation of injury volume in the cerebral hemisphere and caudate nucleus, recovery of SEP amplitude was incomplete in all groups. Incomplete SEP recovery in the ischemia groups treated with NPC 17742 may be attributed in part to the fact that the drug reduced SEP amplitude in nonischemic cats. It is possible that low doses of the drug would not suppress SEP amplitude in nonischemic cats yet would still afford neuroprotection. Because NPC 17742 did not decrease CMRO2, our data do not support the hypothesis that the mechanism of protection is related to an alteration in cerebral oxidative metabolism.

Our data strongly support the hypothesis that excitatory amino acids are important in the mechanism of brain injury after transient focal ischemia in cats. The mechanism of protection afforded by inhibition of the NMDA receptor has yet to be clearly defined. However,
it has been hypothesized that stimulation of the NMDA receptor results in an increase in neuronal calcium concentration, which possibly leads to calcium overload in mitochondria, activation of various phospholipases, increased production of oxygen radicals, and increased production of nitric oxide (NO). The role of calcium in the mechanism of brain damage from focal ischemia is also suggested by the findings that calcium channel blockers alone or in combination with MK-801 reduce damage. In addition, drugs that attenuate excitatory amino acid release during ischemia also are associated with a reduction in brain injury after permanent focal ischemia in rats.

Initial studies of NMDA antagonists in focal ischemia used pretreatment. Later studies showed that treatment during ischemia was effective also when administration of the antagonist was delayed by 2 hours in the case of permanent MCA occlusion in cats or by 30 minutes in the case of 2 hours of transient MCA occlusion in cats. In the present study, we administered the loading dose of the antagonist over a 30-minute period starting 15 minutes before the end of the ischemic period to ensure that a significant amount of drug would be delivered to low-flow areas before reperfusion. The rationale for the paradigm is that treatment with a competitive NMDA antagonist might serve as a useful adjunct to thrombolytic therapy. Our results indicate that inhibition of NMDA receptors during reperfusion provides neuroprotection. This conclusion is supported by the results of Dirnagl et al., who found that the combination of pretreatment and posttreatment was more effective than pretreatment alone in the spontaneously hypertensive rat subjected to focal ischemia, and by the results of Swan and Meldrum, who found that treatment after 15 minutes of global forebrain ischemia was protective of CA1 pyramidal cells. Although one might anticipate that high interstitial glutamate levels during ischemia would result in maximal NMDA receptor activation, the accompanying acidosis may inhibit activation. Restoration of pH during reperfusion and persistent glutamate elevation at the synaptic cleft may lead to NMDA receptor activation during reperfusion. Activation of NMDA receptors is thought to contribute to the propagation of spreading depression waves over cortex during ischemia and to aggravate the imbalance of blood flow to oxygen consumption. Thus, NPC 17742 may act by reducing the incidence of spreading depression during reperfusion.

Toxicity from NMDA can be reduced in cell culture by inhibitors of NO synthase. During ischemia and reperfusion, release of excitatory amino acids may increase NO synthase activity by a mechanism that involves NMDA and non-NMDA glutamate receptors. Because there appears to be an increase in tissue NO levels during early reperfusion, administration of NPC 17742 just before reperfusion might act to reduce postischemic stimulation of NO synthase. The mechanism of injury with NO synthase stimulation during ischemia and reperfusion appears to be related to a combination of NO with superoxide anion generated during reperfusion, producing a much more toxic radical. However, if this be the reason why so much amino acid stimulation in vivo was mediated through increased production of NO, we would anticipate that inhibition of NO synthase activity or administration of superoxide dismutase would produce similar brain protection as produced with NPC 17742. Although we have found previously that both inhibition of superoxide anion production with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and inhibition of NO production with Nω-nitro-L-arginine methyl ester (L-NAME) decrease the extent of injury from focal ischemia in caudate nucleus, neither exerted a significant effect on injury volume in cerebral hemispheres. It is possible that the limited effect of PEG-SOD was due to inadequate penetration of PEG-SOD into the brain. However, it is unlikely that inadequate penetration was the rationale for the limited efficacy of L-NAME because we demonstrated that, at the dose used, L-NAME completely inhibited NO synthase. Because the 83% reduction in cortical injury volume with NPC 17742 at the 5-mg/kg dose was so much more impressive than that with L-NAME or PEG-SOD, we speculate that injury induced by NMDA receptor activation in vivo involves mechanisms in addition to those associated with NO and superoxide anion.

The 83% reduction in cortical injury at the 5-mg/kg dose of NPC 17742 is greater than the 33% to 50% reduction seen with MK-801 in cats and the 65% reduction seen with D-(E)-4-(3-phosphonoprop-2- enyl)piperazine-2-carboxylic acid (CPP-ene) in cats. Furthermore, reduction in caudate injury was not significant in earlier studies with MK-801, whereas striatal protection was observed with dextromethorphan. We observed 49% and 64% reductions in caudate injury at the 5- and 50-mg/kg doses, respectively. The relatively large percentage of reduction in both cortex and caudate injury in our study may be related to differences in the properties of the various NMDA antagonists, to the use of a shorter duration of ischemia in the present study (60 minutes), and to the possibility that TTC staining after 4 hours of reperfusion may not precisely predict the eventual size of a mature infarct. In addition, because we did not perform histology on the salvaged areas, we cannot exclude that there may be significant neuronal loss without pancellular necrosis in areas that stained with TTC.

MK-801 and the competitive NMDA receptor antagonist CGS-19755 were found to improve CBF distribution during transient focal ischemia, possibly by reducing brain swelling. However, others have not found an improved CBF distribution with MK-801 treatment in cats. Our study does not support the hypothesis that NPC 17742 protects brain from injury through a mechanism that involves a favorable redistribution of CBF during ischemia. In fact, NPC 17742 caused an acute reduction in CBF in cats not subjected to ischemia. Although blood flow tended to be higher in NPC-5 cats compared with saline-treated cats during MCA occlusion, blood flow was similar between NPC-50 cats and saline-treated cats. In addition, as compared with saline-treated cats, NPC 17742 causes a distinctive and more favorable shift in the relation of hemispheric injury to end-ischemic CBF. Furthermore, NPC 17742 does not act by reducing baseline CMR2, although as discussed above it may act to decrease transient increases in CMR2 associated with decreases in hemispheric injury by inhibiting cortical spreading depression.

In conclusion, these data support the hypothesis that NMDA receptor activation plays an important role in
the mechanism of acute brain injury after transient focal ischemia. NPC 17742 afforded protection even when administered at the end of ischemia and during reperfusion. The mechanism of protection for NPC 17742 was not related to a redistribution of CBF.

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Cerebral ischemia can cause excessive release of the excitatory neurotransmitter glutamate producing over-stimulation of glutamate receptors and eventually cell death.\textsuperscript{1,2} Accumulation of extracellular glutamate can activate several receptor subtypes including N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors.\textsuperscript{1,2} Brain injury under such conditions appears to be due to excess calcium influx and may be mediated by nitric oxide, formation of oxygen radicals, or other mechanisms.\textsuperscript{1-3} Antagonists of NMDA receptors and antisense oligodeoxynucleotides to NMDA receptor channels protect cultured neurons from excitotoxicity and decrease infarct size after cerebral ischemia.\textsuperscript{1-2} Blockade of AMPA and/or kainate receptors may also be protective during cerebral ischemia.\textsuperscript{1}

The present study examined the hypothesis that the competitive NMDA antagonist NPC 17742 (2R,4R,5S-[2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid]) would reduce brain injury in an experimental model of focal ischemia. The study was systematic and carefully done. The volume of tissue injured in the ipsilateral hemisphere and the caudate nucleus was attenuated by NPC 17742 and was associated with partial recovery of somatosensory evoked potentials. The mechanism of protection did not appear to relate to a redistribution of cerebral blood flow. An important aspect of the study is that intravenous NPC 17742 was protective when administered just before reperfusion (during ischemia).

These data support the concept that overstimulation of NMDA receptors is an important mediator of brain injury after focal cerebral ischemia. Competitive NMDA receptor antagonists such as NPC 17742 may exert side effects that are less adverse, and thus be potentially more useful clinically, than noncompetitive NMDA receptor antagonists such as MK-801.

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