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Combination Therapy with MK-801 and α-Phenyl-*tert*-butyl-nitrone Enhances Protection against Ischemic Neuronal Damage in Organotypic Hippocampal Slice Cultures

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***In vitro* combinations of MK-801, an NMDA receptor antagonist, and α-phenyl-*tert*-butyl-nitrone (PBN), a free radical scavenger, have been tested for possible additive neuroprotective effects against anoxia/hypoglycemia (Ax/Hg)-induced neuronal damage. Rat organotypic hippocampal slice cultures were exposed to Ax/Hg for different lengths of time to vary the severity of the insult. Cell death (CD) was assessed using propidium iodide fluorescence and expressed as a percentage of the total neuronal cells present. Pretreatment with PBN alone (500 μ*M*) provided significant protection against moderate ischemic injury and reduced CD from 65% in controls to 2% in the treated group ($P < 0.003$). A longer ischemic exposure time caused more neuronal damage, which was only slightly reduced by PBN, but significantly reduced by MK-801 (30 μ*M*) (4% CD with MK-801 vs 75% CD in controls; $P < 0.0003$). With a further increase in the time of ischemic exposure, MK-801 was still protective (33% CD with MK-801 vs 90% CD in controls; $P < 0.002$), although the combination MK-801 + PBN was more efficient (7% CD with combination, $P < 0.01$ compared to MK-801 alone). With yet a further increase in the ischemic exposure, PBN or MK-801 alone was not protective; however, a combination of the two still provided significant protection (64% CD with combination vs 100% CD with MK-801 alone; $P < 0.01$). PBN was protective when administered up to 2 h after Ax/Hg (66% CD in controls vs 36% CD with PBN 500 μ*M*; $P < 0.007$). The combination MK-801 + PBN was able to increase the therapeutic window up to 3 h (61% CD in controls vs 41% with PBN alone vs 7% with MK-801 + PBN; $P < 0.002$ compared to PBN alone). In conclusion, the combination of MK-801 and PBN increases both the efficacy and the time window of protection against ischemia.**

INTRODUCTION

The growing knowledge regarding the mechanisms of cellular injury in the brain has stimulated the development of new therapeutic strategies to improve neuronal resistance to ischemia. Combination therapies which can act by blocking several steps of the pathophysiological processes leading to neuronal death constitute a promising way to extend the effect of neuroprotection (13).

Activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors and the production of oxygen free radicals represent major potential targets for therapeutic intervention against ischemic neuronal damage. Recent evidence shows that NMDA receptor activation may lead to the direct generation of superoxide anion ($\cdot O_2^-$) and hydroxyl radical (·OH) (8). Moreover, oxygen radicals can enhance the release of glutamate and inhibit its reuptake and inactivation (33, 35), thus promoting a vicious cycle. NMDA receptor antagonists and free radical scavengers have independently been shown to provide protection against cerebral ischemia in different *in vitro* and *in vivo* models (28, 36, 44, 45). Combined blockade of these pathways therefore constitutes a logical association to increase neuronal resistance to excitotoxic and ischemic injury.

We have tested combinations of MK-801, an NMDA receptor antagonist, and PBN, a free radical spin trapping agent, for possible additive effects against oxygen/glucose deprivation in rat hippocampal slice cultures. We have also examined whether delayed coadministration of MK-801 and PBN can increase the window of efficacy of these agents following ischemia.

METHODS

Preparation of Cultures

Details of the methodology have been described elsewhere (31, 32). Organotypic cultures of the hippocampus were prepared according to the method of Gähwiler (12) as modified by Stoppini *et al.* (42). Hippocampal slices were prepared from 5- to 7-day-old neonatal rats (Sprague–Dawley, Bantin and Kingman Inc., Fremont, CA). After rapid dissection of the brain and transverse section of the hippocampus, individual slices (435 μm) were placed on 28-mm sterile transparent Anocel membranes (Whatman Inc., Clifton, NJ) and

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grown for 10–14 days in culture trays with 1.5 ml growth medium consisting of 50% MEM (Gibco Laboratories, Grand Island, NY) supplemented with Hepes and sodium bicarbonate, 25% Hank's balanced salt solution, 25% horse serum, and glucose to a final concentration of 6.5 mg/ml. The cultures were grown at 36.5°C, 90–100% humidity, and 5% CO_2. Propidium iodide (PI) (Sigma Chemical, St. Louis, MO), which rapidly enters cells with damaged membranes and becomes brightly fluorescent after binding to nucleic acids, was added (0.5 μg/ml) to the culture medium as an indicator of neuronal death during the experiments. Animals were cared for according to the guidelines of the University of Washington animal care committee.

Preparation of Compounds

The NMDA antagonist MK-801 was obtained from Research Biochemicals Inc., Natick, Massachusetts. PBN was obtained from Sigma Chemical. Compounds were added to the medium as small aliquots in glucose-free HBSS to achieve the final concentrations described. An equivalent amount of HBSS was added to control cultures. Depending on the protocol used, compounds were administered to the cultures either before or at increasing time intervals following the ischemic insult.

Induction of Neuronal Damage

For induction of neuronal damage, cultures were rinsed 3× in glucose-free HBSS and then placed in 1.5 ml of HBSS without glucose. All HBSS solutions were adjusted to the same molarity as the growth medium with sucrose. The temperature of each individual culture well was maintained at 37°C with an electronic thermometer and carefully controlled to be equivalent in all cultures to ensure that the results were independent of temperature fluctuation. The cultures were then transferred into an anaerobic chamber (Forma Scientific) preequilibrated to 37°C with an atmosphere of 0% oxygen, 10% hydrogen, 5% CO_2, and 85% nitrogen. The hydrogen was present for interaction with a palladium catalyst that maintained the oxygen concentration at 0%. Increasing levels of neuronal damage were obtained in the control cultures by extending the duration of exposure to Ax/Hg (from 28 min to 50 min). Upon removal from the anaerobic chamber, the cultures were transferred to prewarmed growth medium containing PI and placed in a CO_2 incubator at 36.5°C for 48 h.

Assessment of Cell Death by Image Analysis Using PI

The cultures were examined using a Nikon Diaphot inverted fluorescent microscope. Fluorescent images were obtained using a Dage 72 CCD camera (Michigan City, IN) and were digitized using Optimas image analysis software (Bio-Scan Inc., Edmonds, WA). The intensity of PI fluorescence in the CA1 area was used as an index of cell death. The first measurement of fluorescent intensity was performed 40 to 48 h after initial exposure. The remaining neurons were killed by exposing the cultures to 3 h of anoxia. The fluorescent intensity obtained 24 h after 3 h of anoxia was set equal to 100% damage and was then compared to the fluorescent intensity following the initial insult. The integrated gray values (fluorescent intensity) from CA1 following the initial insult were then expressed as a percentage of the value obtained following 3 h of anoxia. The values were averaged for each group and expressed as mean ± SEM. Statistical analysis of the results was performed using the Mann–Whitney test with Bonferroni correction for multiple comparisons. Differences were considered significant at a P value < 0.05.

RESULTS

Neuroprotective Effects of PBN

When administered before oxygen/glucose deprivation, PBN alone was able to provide significant protection against a moderate ischemic insult. After 30 min exposure to artificial ischemia producing 65% cell death in the control cultures, PBN showed a dose-dependent ability to reduce cellular injury, with the maximum at a concentration of 1000 μ*M* (Fig. 1A). The dose–response curve of PBN was characterized by an "all-or-nothing" effect, evident between 10 and 100 μ*M*. Figure 1B gives an example of the significant neuroprotective effect provided by PBN. Based on these results and previous studies with MK-801 in our preparation (32), concentrations of 500 μ*M* PBN and 30 μ*M* MK-801 were used for combination experiments.

Combinations of PBN and MK-801 Administered before Oxygen/Glucose Deprivation

Increasing the exposure time to the ischemic insult was used to test combinations of PBN and MK-801. At 75% cell death in controls, PBN provided no significant protection, whereas MK-801 alone or the combination MK-801 + PBN was fully protective (Fig. 2A). At 90% cell death in controls, MK-801 alone was still partially protective, although the combination MK-801 + PBN was significantly more efficient ($P < 0.01$) (Fig. 2B). When 100% cell death was produced in controls and in the groups treated with PBN or MK-801 alone, the combination was still able to significantly reduce cellular injury ($P < 0.01$) (Fig. 2C).

Post-ischemic Administration of PBN and MK-801 Alone or in Combination

When administered at a fixed concentration of 500 μ*M* at increasing time intervals following anoxia/hypoglycemia, PBN alone was able to significantly

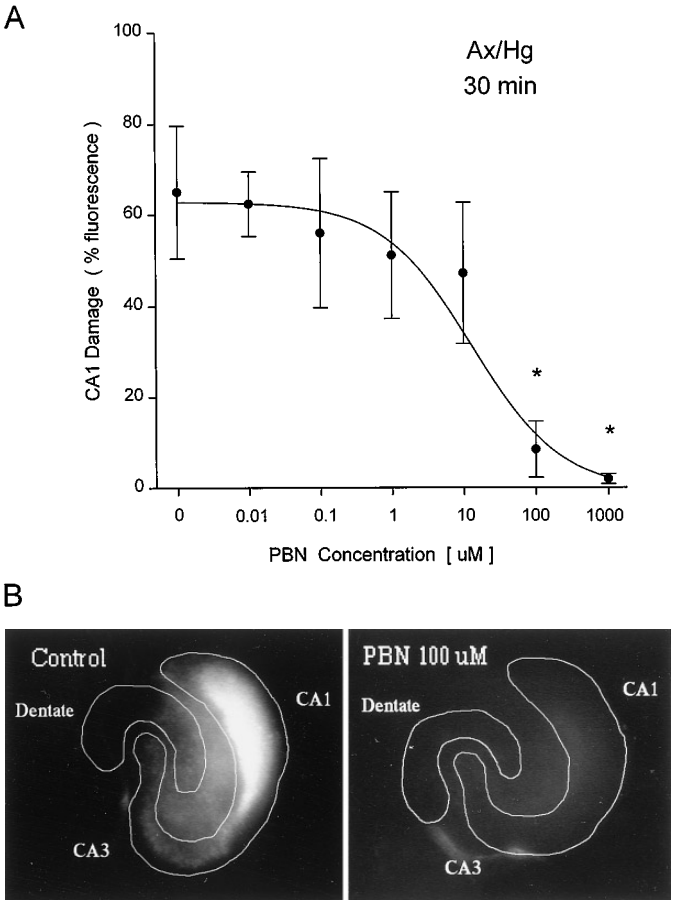

FIG. 1. (A) Dose–response curve showing the effect of increasing concentration of PBN on reducing propidium iodide fluorescence in CA1 caused by oxygen and glucose deprivation for 30 min. Note the "all-or-nothing" protective effect between 10 and 100 μ*M* of PBN. $n = 10$ for each data point. $^{*}P < 0.003$ compared to control using Mann–Whitney test with Bonferroni correction for multiple comparisons. (B) Illustration of propidium iodide fluorescence in a control hippocampal culture (left) and in a culture treated with 100 μ*M* PBN (right) 48 h after 30 min of anoxia/hypoglycemia. Marked fluorescence in the control indicates postischemic cell death. Significant decrease of fluorescence in the treated culture indicates neuroprotection.

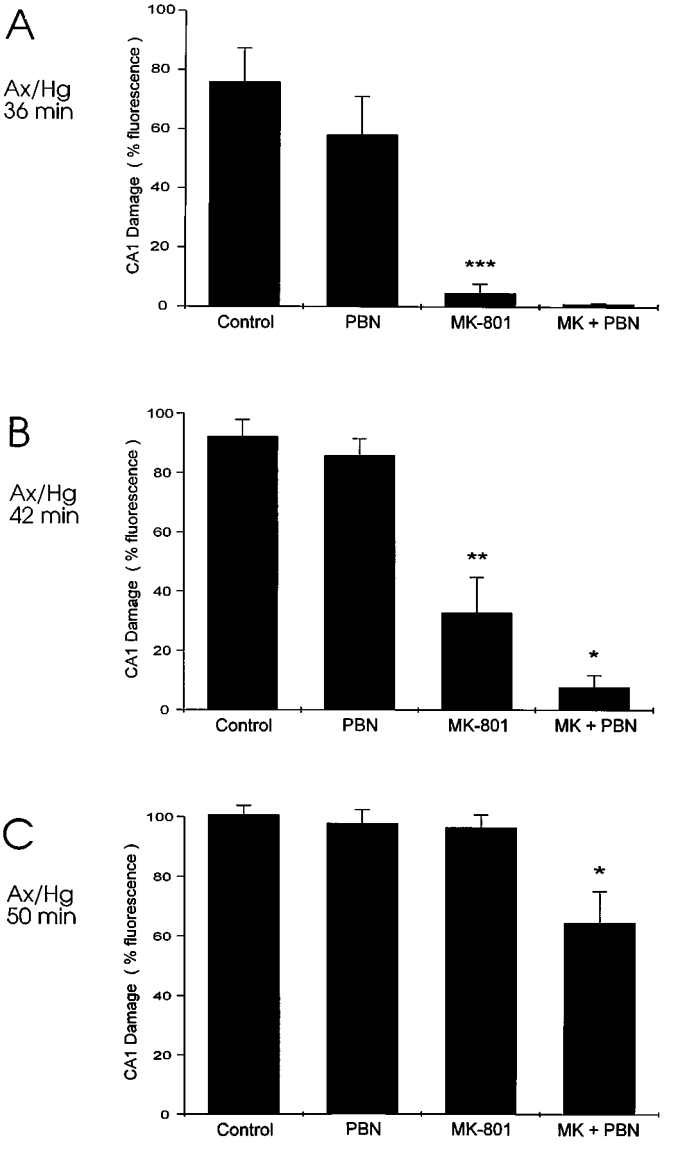

FIG. 2. Illustration of the neuronal protection provided by PBN (500 μ*M*), MK-801 (30 μ*M*), and the combination PBN + MK-801 (same concentrations) against increasing ischemic damage in CA1. (A) After 36 min of anoxia/hypoglycemia producing 75% cell death in control cultures, PBN did not provide significant neuroprotection, whereas MK-801 alone was as effective as the combination PBN + MK-801. (B) After 42 min of anoxia/hypoglycemia producing 90% cell death in control cultures, MK-801 alone was still able to provide statistically significant, although partial, neuroprotection, whereas the combination PBN + MK-801 provided significantly more protection than MK-801 alone. (C) After 50 min of anoxia/hypoglycemia producing 100% cell death in control cultures and in cultures treated with PBN or MK-801 alone, the combination was still able to provide statistically significant neuroprotection. $n = 10$ for each group. $^{*}P < 0.01$ (compared to MK-801 alone); $^{**}P < 0.002$ (compared to control); $^{***}P < 0.0001$ (compared to control) using Mann–Whitney test with Bonferroni correction for multiple comparisons.

reduce cellular death up to 2 h in a time-dependent fashion (Fig. 3A). At 3 h, PBN provided partial, although not significant, protection. MK-801 alone was not protective if not given immediately after the ischemic injury (Fig. 3B). The combination MK-801 + PBN was able to increase the therapeutic window up to 3 h (Fig. 4). At 6 h following the ischemic insult, neither PBN alone nor the combination MK-801 + PBN was effective (results not shown for the combination).

DISCUSSION

Our results demonstrate that the combined administration of an NMDA receptor antagonist and a free radical scavenger enhances neuroprotection against oxygen and glucose deprivation in organotypic hippo-

campal slice cultures. The combination of MK-801 and PBN significantly improved neuronal survival to increasing ischemic injury and was able to provide neuroprotection at injury levels where each agent individually was ineffective. In addition, the combination significantly extended the therapeutic window up to 3 h after ischemia.

The rationale for this association is based on recent evidence that links excitotoxicity with the production of oxygen free radicals. A direct generation of superoxide ($\cdot O_2^-$) following exposure to NMDA has been demonstrated in cultures of cerebellar neurons using electron paramagnetic resonance (21). *In vitro* studies have demonstrated that free radical scavengers can attenuate neuronal damage caused by direct exposure to glutamate, kainate, or NMDA (10, 11, 21, 26). More recently, both kainate and NMDA have been shown to increase free radical generation *in vivo* (17, 43). There is also evidence that free radical scavengers can protect neurons *in vivo* against direct excitotoxic injury (39).

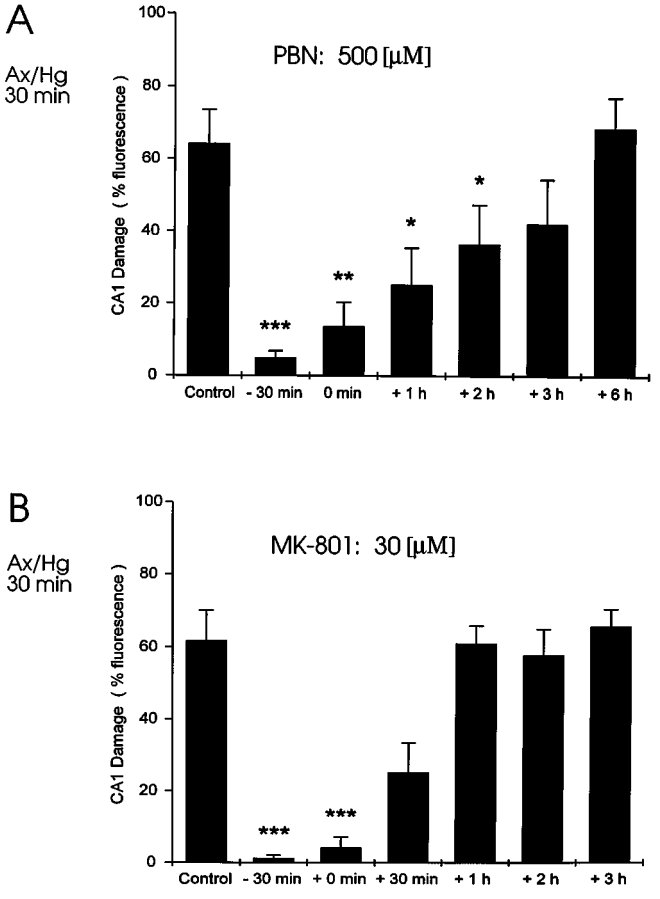

FIG. 3. Illustration of the neuronal protection provided by PBN (500 μ*M*) and MK-801 (30 μ*M*) when administered before (−30 min), immediately after (0 min), and with increasing delay (1 to 6 h for PBN; 30 min to 3 h for MK-801) following 30 min of anoxia/hypoglycemia. (A) PBN alone was able to provide statistically significant protection up to 2 h after ischemia, whereas (B) MK-801 showed no significant protective effects if not given immediately after the ischemic injury. $n = 10$ for each group. $^*P < 0.007$; $^{**}P < 0.001$; $^{***}P < 0.0001$ (compared to control) using Mann–Whitney test with Bonferroni correction for multiple comparisons.

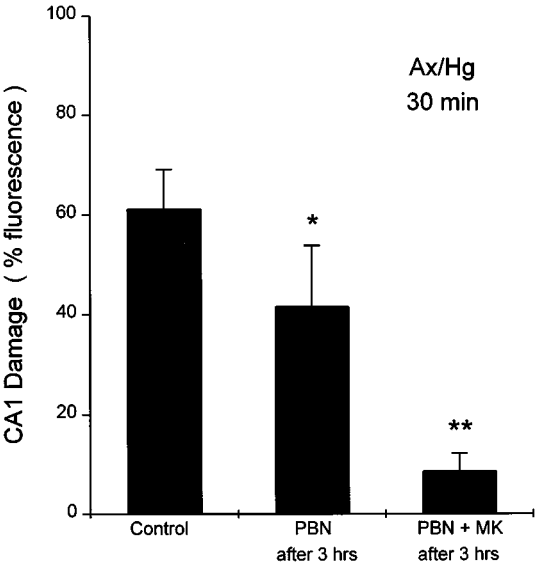

FIG. 4. Illustration of the extended therapeutic window provided by the combination PBN + MK-801 against ischemic injury (PBN, 500 μ*M;* MK-801, 30 μ*M*). Three hours after 30 min of anoxia/hypoglycemia, the administration of PBN alone was unable to provide statistically significant neuronal protection, whereas the combination significantly reduced neuronal death compared to control cultures and to cultures treated with PBN alone. n = for each group. $^*P < 0.16$ (compared to control); $^{**}P < 0.002$ (compared to PBN alone) using Mann–Whitney test with Bonferroni correction for multiple comparisons.

Free radicals have been implicated in the pathogenesis of a large spectrum of diseases and are currently considered as major causative agents of postischemic injury in a variety of tissues, including heart, intestine, and the central nervous system (27, 38). Free radical production has been detected in the course of ischemia in rat, gerbil, and pig brains with a dramatic burst at onset of reperfusion (2, 23, 33, 41). Oxygen free radicals produced in excess can attack all major components of cells, leading to loss of function and structural damage. Lipid membranes of neurons are particularly vulnerable to oxygen radical damage because of their high content in polyunsaturated fatty acids, which are easily oxidized by toxic species such as ·OH (14).

During ischemia, activation of NMDA receptors secondary to excessive release of excitatory amino acids has been shown to produce a massive entry of calcium into cells and to initiate a series of intracellular events which finally lead to neuronal death. Among other deleterious effects, elevation of intracellular calcium can promote the uncontrolled generation of oxygen radicals by a number of different pathways. The Ca^{2+}-dependent activation of phospholipase A_2 releases arachidonic acid, leading to the production of $\cdot O_2^-$ through

its further metabolism by cyclo-oxygenase and lipoxygenase (6, 9, 21). Xanthine dehydrogenase is enzymatically converted to xanthine oxidase following intracellular Ca^{2+} elevation and catabolizes purine bases with the production of $\cdot O_2^-$ (27). This reaction may be exacerbated by energy depletion and AMP accumulation. Furthermore, lactic acidosis favors the liberation of Fe^{2+} from intracellular stores, which promotes the formation of $\cdot OH$ from H_2O_2 (15). Elevated intraneuronal Ca^{2+} has also been shown to activate the enzyme NO-synthase with subsequent production of NO (29). NO then interacts with $\cdot O_2^-$ to yield the peroxynitrite anion ($ONOO^-$), which decomposes to $\cdot OH$ (1).

PBN is a diamagnetic compound that reacts with unstable oxygen radicals to form relatively stable adducts with a typical electron spin resonance signal. PBN has been widely used to detect free radical production and metabolism in biological systems (18, 19, 22). In addition, PBN has recently been shown to exert remarkable protective effects on ischemia/reperfusion injury of the brain (4, 7, 36, 44). In rats subjected to focal cerebral ischemia with or without reperfusion, PBN was able to reduce infact size to 50% of controls or less (3, 45). Noteworthy was the ability of PBN to provide significant delayed protection for up to 12 h after permanent occlusion of the middle cerebral and ipsilateral common carotid arteries (3). Because PBN's action of trapping free radicals neutralizes them, it probably acts as a true free radical scavenger capable of interrupting the cascade of intracellular events that lead to oxidative tissue damage (4). In addition to ischemia/reperfusion injury, PBN has been shown to provide protection in other situations where free radicals are thought to be primarily involved, such as traumatic brain injury, age-related oxidative changes of the brain, and exposure to endotoxin or paraquat (5, 16, 37, 40).

Noncompetitive and competitive antagonists of the NMDA receptor are currently in advanced development for the treatment of stroke and traumatic brain injury and many drugs are now entering clinical trials (30). In order to increase their efficacy and possibly reduce their side effects, NMDA receptor antagonists have been studied with variable success in several types of therapeutic combination, including calcium-channel blockers, 21-aminosteroids, antimuscarinic agents, growth factors, hypothermia, thrombolysis, and moderately increased acidity (13). Promising results have been obtained with NMDA antagonists associated with non-NMDA antagonists against oxygen/glucose deprivation or glutamate toxicity *in vitro* and against focal and global ischemia *in vivo* (20, 24). The combination of MK-801 with the GABA-A agonist muscimol has been shown to increase brain protection in rats submitted to embolic stroke (25). This combination could possibly enhance the clinical utility of NMDA antagonists because muscimol and other GABAergic drugs such as diazepam and barbiturates are known to prevent neuronal vacuolation that arises in the cingulate and retrosplenial cortex following treatment with NMDA antagonists (25, 34). In a recent study using intrastriatal excitotoxin injections in rats, MK-801 associated with the free radical scavenger S-PBN produced additive protective effects against malonate and 3-acetylpyridine toxicity (39).

One important aspect of combination pharmacotherapies for stroke or traumatic brain injury is the possibility of increasing the therapeutic window of NMDA antagonists. Lyden *et al.* (25) found that muscimol combined with MK-801 still provided significant protection at lower doses up to 1 h in their rat model of embolic stroke. Our findings also suggest that the combination of MK-801 with PBN can extend the efficacy of both agents for several hours after the ischemic injury.

In conclusion, this study demonstrates that *in vitro* combination of an NMDA receptor antagonist and a free radical scavenger increases both the efficacy and the therapeutic window of these agents. These results furnish a cellular basis for combined treatment of cerebral ischemia and support further *in vivo* and clinical studies of combination therapy in neuroprotection.

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