The Neuroprotective Effect of the Novel AMPA Receptor Antagonist PD152247 (PNQX) in Temporary Focal Ischemia in the Rat

Gerald P. Schielke, PhD; Nancy C. Kupina, BS; Peter A. Boxer, PhD; Christopher F. Bigge, PhD; Devin F. Welty, PhD

Background and Purpose—Evidence suggests that glutamate contributes to ischemic brain damage through activation of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor. We tested the novel, selective AMPA receptor antagonist PD152247 (PNQX) in a model of temporary focal ischemia to determine the dose-response relationship and to investigate the contribution of drug-induced hypothermia to the neuroprotective action of AMPA receptor antagonists.

Methods—Temporary focal cerebral ischemia was induced in Sprague-Dawley rats by occluding the middle cerebral artery and both carotid arteries for 3 hours. Body temperature was monitored by telemetry. PNQX was administered intraperitoneally or by intravenous infusion with various doses for 6 hours. Lesion volume was determined after 3 days by stereological methods.

Results—PNQX reduced the lesion volume by 51% after intraperitoneal administration. The intravenous dose-response study demonstrated that the lowest effective dose of PNQX was 1.0 mg/kg per hour, which corresponded to a steady state plasma level of 685 ng/mL. Neuroprotection was demonstrated at PNQX plasma concentrations that did not lower body temperature over the entire course of the experiment.

Conclusions—AMPA receptor activation plays an important role in the development of ischemic brain damage. Thus, novel AMPA receptor antagonists may be useful for the treatment of stroke in humans. (Stroke. 1999;30:1472-1477.)

Key Words: cerebral ischemia, focal ■ glutamate antagonists ■ middle cerebral artery occlusion ■ neuroprotection ■ rats

Elevation of extracellular glutamate after cerebral ischemia is thought to play a major role in the pathophysiological processes leading to death of ischemic brain tissue. Early studies demonstrated that glutamate killed neurons in culture by a calcium-dependent process that was blocked by selective antagonists of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), the ligand for a distinct non-NMDA glutamate receptor subtype, also results in cell death in cultured neurons with a slower time course. The observation that extracellular brain glutamate levels, measured by microdialysis, are increased in ischemic tissue suggested that the excitotoxicity demonstrated in culture may be relevant to ischemic cell death in vivo. Subsequently, it was demonstrated that blockers of the NMDA receptor reduce brain damage in several animal models of cerebral ischemia.

More recently, several antagonists of the AMPA subtype of the glutamate receptor have been discovered and shown to be neuroproprotective in a variety of models of cerebral ischemia.2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), a quinoxalinedione, was the first selective, competitive AMPA antagonist demonstrated to be effective in reducing ischemic brain damage. Unlike NMDA receptor antagonists, the AMPA receptor antagonist NBQX reduces selective neuronal death after global forebrain ischemia. In models of focal ischemia, AMPA antagonists including NBQX and more recently discovered antagonists LY215490, YM90K, YM872, and GYKI52466 have been reported to reduce the volume of necrotic tissue. Additional evidence that AMPA receptor activation plays a role in ischemia is the recent finding that transgenic mice that
overexpress the AMPA receptor subunit GluR2-flip have increased ischemic brain damage.24

Recently, a novel quinoxalinedione PD152247 (PNQX) was discovered.25 PNQX binds with high affinity to the AMPA receptor (IC50, 90 nmol/L) and with modest affinity to the kainate receptor and the glycine site of the NMDA receptor. PNQX antagonizes effects of AMPA in AMPA-induced cytotoxicity in primary neuronal cultures and protects against AMPA and maximal electroshock-induced seizures in mice (unpublished data, Boxer and Probert, 1999).

The present study was performed to determine whether PNQX is neuroprotective in a model of temporary focal ischemia. The first rationale for this study is that evaluation of novel AMPA antagonists with varying profiles of in vitro and in vivo activity and water solubility is important because the solubility-related renal toxicity of NBQX18 has prevented its clinical testing. Second, since it has been suggested that drug-induced hypothermia is responsible for the neuroprotective effects of AMPA antagonists,26 the possible contribution of hypothermia to the neuroprotective action of PNQX was addressed. Finally, the relationship between plasma concentrations of PNQX and the degree of neuroprotection in this model was evaluated.

Materials and Methods

All procedures were within the guidelines of the Institutional Animal Care and Use Committee of Parke-Davis.

Surgery

Male Sprague-Dawley rats (Charles River Laboratories), weighing 275 to 325 g, were anesthetized with 2% isoflurane (Anaquest) balanced with air, oxygen and allowed to breathe spontaneously. Rats receiving intraventricular treatments had femoral vein catheters (Micro-Renathane, Braintree Scientific) implanted before middle cerebral artery occlusion (MCAO). Both common carotid arteries were isolated through a small neck incision and occluded with vascular clips (Microserrefine No. 3, FST Inc) just before MCAO. A vertical incision was made between the left eye and ear, the temporalis muscle was partially excised, and a craniotomy was performed at the site where the middle cerebral artery (MCA) meets the rhinal fissure. The dura over the MCA was opened and reflected to allow occlusion of the vessel with a Sundt AVM Micro Clip No. 1 (Codman) at the point where it meets the rhinal fissure. Anesthesia was discontinued until the time of reperfusion. All vascular clips were removed after 3 hours of ischemia, and reperfusion of the MCA was verified by examination of the site of occlusion. An absorbable gelatin sponge (Gelfoam, Upjohn) was placed in the area of excised brain, and their brains were removed and frozen in isopentane over dry ice at −40°C and stored at −80°C.

Temperature Monitoring

Rats receiving intraperitoneal treatments (study 1) were placed in isolators after MCAO and kept warm with a heating pad for 5 hours. In study 2, body temperature was maintained at 37.5°C during surgery, drug infusion, and recovery (6 hours) by individual feedback controllers and heating pads. In both studies, after the rats were returned to their cages, core body temperatures were monitored by telemetry with the use of a temperature-sensing radio transmitter (Data Sciences International) previously placed in the peritoneal cavity. The radio signal was converted to body temperature (Datquest IV 2.10, Data Sciences International) and recorded every minute for 72 hours. Temperatures were averaged over 1-hour intervals for analysis and plotted at 5-hour intervals for illustration.

Drug Delivery

In study 1, drug or vehicle was administered by 3 intraperitoneal injections given at 0.5-hour intervals beginning 30 minutes after initiation of ischemia. The dose of PNQX was 30 mg/kg × 3 injections (total of 90 mg/kg) (concentration, 15 mg/mL; volume, 2 mL/kg per injection). PNQX was suspended in water, and pH was adjusted with methanesulfonic acid. In study 2, drug or vehicle was administered by continuous intravenous infusion (3 mL/kg per hour) for 5 hours, beginning 15 minutes after the initiation of ischemia. PNQX was dissolved in water with a small amount of 85% lactic acid; the pH was adjusted to 4.0 with sodium hydroxide and filtered before administration. Rats were randomly assigned to 1 of 5 groups: PNQX at 5.0, 2.5, 1.0, or 0.1 mg/kg per hour or vehicle.

Histology

Brains were sectioned at −17°C on a cryomicrotome. Twenty-micrometer coronal sections were collected at 600-μm intervals and stained with hematoxylin and eosin. Lesion volume was measured with the aid of the stereological software package GRID (Olympus, Denmark AS). A grid of points was randomly superimposed over a video image of each tissue section, and the number of points overlaying an area of interest was counted. Total lesion volume was estimated by the equation V = t × A(p) × ΣP, where V is lesion volume, t is the distance between sections analyzed (1200 μm), A(p) is the surface area associated with 1 grid point, and ΣP is the total number of grid points associated with an area of interest in all of the sections examined.

PNQX Analytical Methods

Plasma PNQX levels were determined by UV high-performance liquid chromatography. In brief, matrix standards, quality control samples, and unknown samples were mixed with internal standard, diluted with 0.1 mol/L sodium phosphate buffer (dibasic), pH 6.0, and extracted through C18 solid-phase cartridges with 1% trifluoroacetic acid in acetonitrile. Samples were then evaporated to dryness and reconstituted with mobile phase and injected onto a reverse-phase C3 column. The mobile phase consisted of 90% 20 mmol/L sodium phosphate (dibasic), pH 2.75, and 10% methanol. Detection was by ultraviolet absorption at 210 nm, and quantitation was by peak height ratios.

Statistical Methods

Data are expressed as mean ± SD. Lesion volumes were compared by ANOVA and a Tukey-Kramer multiple comparisons test. Temperature data from study 1 were analyzed with 1-way ANOVA to compare control with the drug-treated animals at each time point. In study 2, ANOVA with linear contrasts to test for a relationship between dose and temperature was performed. P < 0.05 after correction for multiple comparisons was considered significant.

Results

Effect of PNQX on Infarct Volume

In this model of temporary focal ischemia, the histological lesion, defined by the area of pallor at 72 hours, is large and primarily limited to the cortical tissue. In study 1, rats were treated intraperitoneally with PNQX (30 mg/kg at 30, 60, and 90 minutes after MCAO), a dosing paradigm used in the original studies with NBQX. The drug-treated group had a mean lesion volume 51% smaller than vehicle-treated rats (287 ± 110 versus 142 ± 87 mm3; P < 0.001) (Figure 1, top). When lesion volumes were calculated by the indirect method,27,28 a similar reduction was seen (171 ± 72 versus 73 ± 57 mm3; P < 0.001), demonstrating that the effect of
PNQX on stroke size was not due solely to a reduction in brain swelling. There was a modest (1°C to 1.25°C) reduction in body temperature, which was statistically significant between 4 and 19 hours after reperfusion in the PNQX-treated rats (Figure 1, bottom).

Relation of PNQX Plasma Concentration to Infarct Reduction
To determine the relationship between plasma levels of PNQX and its neuroprotective action, a 5-hour intravenous dose-response study was performed. Rats treated with PNQX at 5, 2.5, or 1 mg/kg per hour but not 0.1 mg/kg per hour had significantly smaller lesions than vehicle-treated controls (Figure 2, top). Figure 2 (bottom) illustrates that there was a slight but statistically significant reduction in body temperature (0.5°C) between 2 and 6 hours; \( P < 0.05 \) in rats receiving 5 mg/kg per hour PNQX. At the other neuroprotective doses of PNQX (2.5 and 1 mg/kg), body temperatures were not reduced compared with vehicle-treated rats at any time over the entire course of the experiment. Thus, in this reperfusion model of focal ischemia, the neuroprotective action of PNQX is not due to hypothermia. The plasma concentration of PNQX at the end of the 5-hour infusion was 685 ± 534 ng/mL at the lowest neuroprotective dose (1.0 mg/kg per hour), and values for the other groups were roughly proportional to dose. The Table shows the concentrations of PNQX in the plasma...
Discussion

This study demonstrates that treatment with the AMPA receptor antagonist PNQX results in a dose-dependent reduction in cortical infarction in a rodent model of transient focal ischemia. Importantly, PNQX is neuroprotective at plasma drug concentrations that do not lower body temperature.

Previous studies with both competitive antagonists and noncompetitive allosteric modulators of the AMPA receptor have established the neuroprotective potential of blocking the action of this ionotropic glutamate receptor. The quinoxalinedione NBQX has been shown to reduce lesion size in both permanent and temporary rat focal ischemia models. More recently, a 31% reduction in infarct volume in the rat was reported after intravenous administration of the decahydroidosquinoline AMPA receptor antagonist LY-215490. LY-215490 is the racemate of LY-243558, which was found to be neuroprotective in an acute (6-hour) cat MCAO study. YM-90K, another quinoxalinedione, was similarly efficacious in cats under conditions in which physiological variables, including brain temperature, were monitored or controlled. The noncompetitive AMPA antagonist GYKI-52466 reduced the ischemic lesion volume by 68% and 48% when given immediately or 1 hour after MCAO. Unlike NMDA antagonists, AMPA antagonists also reduce selective neuronal loss in global forebrain ischemia models. Interestingly, in this setting NBQX is protective when administration is delayed for up to 12 hours after the ischemic period. However, in focal ischemia, the therapeutic time window for AMPA antagonists has not been well defined. Both NBQX and PNQX (data not shown) are effective when given intraperitoneally 90 minutes after initiation of MCAO. A recent report suggests that the window could be as long as 5 to 6 hours for this class of compounds.

The mechanisms by which AMPA antagonists provide neuroprotection in models of cerebral ischemia are not completely understood. One possibility is that reduced lesion size is secondary to drug-induced hypothermia, since reduction in body temperature in rodents can ameliorate ischemic neuronal damage in both focal and global models. In a study of global forebrain ischemia in the gerbil, NBQX given at 30 mg/kg (×3) caused mild (1°C to 1.5°C) hypothermia that persisted for days. When the postischemic temperature was controlled, neuroprotection was lost. In focal ischemia in rats, mild to moderate hypothermia (30°C to 32°C) reduces lesion volume when the body temperature is lowered during the ischemia (in permanent MCAO), as well as during the reperfusion phase (in temporary MCAO). Typically, studies that have evaluated the neuroprotective effects of AMPA receptor antagonists (see above) used body temperature monitoring and control only during the surgical procedure and drug administration, leaving open the possibility that body temperature was reduced during the remainder of the survival period. In our study, we first demonstrated that a high dose of PNQX administered into the peritoneal cavity reduced lesion volume but also lowered body temperature up to 1.25°C for an extended time. This is likely due to prolonged high-plasma concentrations of PNQX resulting from this dosing paradigm (data not shown). To address the possibility that this extended mild hypothermia might be responsible for the smaller lesions, we performed an intravenous dose-response study in which we controlled body temperature during the 5-hour drug infusion and monitored temperature by telemetry every minute for 67 hours (until death). Body temperature was slightly but significantly reduced for 2 to 6 hours after the drug infusion in rats receiving the highest PNQX dose (5 mg/kg per hour). However, body temperature in rats treated with the 2 lower efficacious doses did not differ from vehicle-treated rats at any time during the experiment. Thus, even though PNQX can cause hyperthermia at high doses, the reduction in infarct volume by PNQX at 1.0 and 2.5 mg/kg per hour cannot be a result of even mild or prolonged hypothermia. Data from acute studies in cats are consistent with the conclusion that AMPA antagonists can be neuroprotective without inducing hypothermia.

AMPA receptor antagonists may protect the brain by reducing neuronal depolarization in the ischemic territory and thus limiting calcium entry into neurons. Excessive glutamate acting on the AMPA receptor depolarizes neurons, and in this depolarized state, the magnesium block of the NMDA channel is lost. Thus, calcium may enter neurons through the open NMDA channels and voltage-dependent calcium channels, as well as the subtype of AMPA-gated channels, which are permeable to calcium. The heteromeric AMPA receptor, which does not contain a GluR2 subunit, has increased calcium permeability. After transient forebrain ischemia, the expression of GluR2 mRNA is reduced in vulnerable brain regions, suggesting a shift to the more calcium-permeable receptors. Excessive intracellular calcium activates pathological processes that contribute to the death of the neurons. Additionally, evidence is accumulating that repeated waves of depolarization in the periphery of the ischemic zone (cortical spreading depressions), which occur after the onset of focal ischemia, increase metabolic demand and contribute to the subsequent loss of viable tissue. NBQX has been shown to reduce the number of these direct current shifts, even though, unlike NMDA antagonists, it has no effect on chemical or electrical invoked spreading depressions.

In summary, AMPA receptor blockade is a therapeutic strategy that is, with few exceptions, robust across several models, laboratories, and chemical classes and that holds promise of a clinically relevant time window. The present study demonstrates that the novel AMPA receptor antagonist PNQX ameliorates focal ischemic brain damage at plasma concentrations that do not produce hypothermia. This finding supports the concept that selective AMPA receptor antagonists will be useful for the treatment of stroke in humans.

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References


This study reports that the new AMPA receptor inhibitor PNQX, administered in the initial postischemic period, reduces cerebral ischemic injury in a rat model of transient focal cerebral ischemia. Importantly, the effect occurs at doses that do not induce hypothermia. The data provide additional evidence in support of the idea that AMPA receptor inhibitors are promising candidates for the treatment of human stroke.

The mechanisms of the protective effect of PNQX remain unclear. Although PNQX is likely to act by blocking AMPA receptors, the cellular mechanisms linking AMPA receptor blockade to neuroprotection have not been defined. Central to the glutamate hypothesis of cerebral ischemic damage is that activation of glutamate receptors increases intracellular calcium, which in turn initiates a series of cytoplasmic and nuclear events leading to tissue damage. Unlike NMDA receptors, however, most AMPA receptors are not permeable to calcium in the normal state. Yet the toxicity mediated through AMPA receptors seems to be calcium dependent. One possibility is that ischemia, through downregulation of the GluR2 subunit of the receptor, leads to formation of calcium-permeable AMPA receptors (eg, Reference 5). This attractive hypothesis, however, needs to be tested experimentally in this particular model.

There are a number of practical issues that still remain to be addressed. First, the “time window” after ischemia in which PNQX administration is still effective remains to be defined. In the accompanying study PNQX was administered 15 or 30 minutes after induction of ischemia, whereas most stroke patients reach emergency rooms several hours after the onset of symptoms. Therefore, it would be important to study the effect of PNQX with longer intervals between induction of ischemia and onset of treatment. Second, it remains to be determined whether PNQX is also effective in species phylogenetically closer to humans. Many treatment modalities reduce stroke volume in rodents but not in higher species.

Therefore, demonstration of efficacy in other species would increase the likelihood that the drug will be effective in humans. Third, it remains to be determined whether effective concentrations of PNQX can be safely reached in humans. These issues will have to be addressed in future studies. Overall, the well-controlled study of Schielke et al provides new and interesting data that, due to their potential clinical applicability, are likely to generate a great deal of interest.

Costantino Iadecola, MD, Guest Editor
Department of Neurology
University of Minnesota
Minneapolis, Minnesota

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