

Kynurenate Inhibition of Cell Excitation Decreases Stroke Size and Deficits

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Pharmacological inhibition of excitatory neurotransmission attenuates cell death in models of global ischemia/reperfusion and hypoglycemia. The current investigations extend these observations to a model of focal ischemia. Kynurenic acid, a broad-spectrum antagonist at excitatory amino acid receptors, was used as treatment (300 mg/kg; 3 doses at 4-hour intervals) before and after focal cerebral ischemia in rats ($n = 54$). Preischemia but not 1 hour postischemia treatment with kynurenate attenuated infarction size ($p < 0.001$) and improved neurological outcome ($p < 0.001$) studied at 24 hours after injury. These data support the role of excitatory neurotransmission in acute neuronal injury and support pharmacological inhibition of cell excitation as a potential therapy for stroke.

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Recently, considerable interest has focused on the role of excitatory neurotransmission, possibly mediated by glutamate and aspartate, in various disease states of the central nervous system [1-3]. Glutamate has been implicated in epileptic brain damage, potentially in the pathophysiology of Huntington's disease, and other neurodegenerative disorders [4, 5]. Our recent studies provide evidence that the selective blockade of cell excitation at the *N*-methyl-D-aspartate (NMDA) receptors using 2-amino-7-phosphonoheptanoic acid (AP-7) protects against hippocampal neuronal injury in global ischemia [6]. Systemic AP-7 administration produces similar results [7]. Wieloch [8] demonstrated AP-7 attenuation of hypoglycemia-induced neuronal damage in striatum; we have shown similar results in hippocampus [9].

We now report the effect of inhibition of excitatory amino acid neurotransmission (using the broad-spectrum agent kynurenate, which blocks excitation at NMDA and non-NMDA receptors) upon the neuropathological and functional outcome of permanent focal cerebral ischemia in the rat.

Materials and Methods

Surgical Procedure

The experimental procedure followed guidelines set by the National Institutes of Health and was approved by the University of California, San Francisco Animal Experimentation

Committee. Fifty-four adult male Sprague-Dawley rats weighing 350 to 400 gm were anesthetized with intraperitoneal chloral hydrate in normal saline (35 mg/100 gm body weight). Polyethylene catheters (25 cm long) were introduced into the left femoral artery for monitoring mean arterial blood pressure (MABP). Normal body temperature was maintained with a heating pad.

The left temporoparietal region was shaved. Rats were placed in the lateral position, and a 2-cm curved vertical incision was made midway between the lateral margin of the left orbit and the external auditory canal. The temporalis muscle was elevated from the skull and the inferotemporal fossa was exposed. Using microsurgical techniques, a 5-mm craniectomy was made in the inferotemporal fossa using a saline cooled dental drill; care was taken not to damage the zygomatic bone or the mandibular nerve. The dura was opened through a stellate incision and the middle cerebral artery (MCA) was exposed. With bipolar coagulation, the MCA was occluded from the origin at the carotid artery continuously up to the point where it is crossed by the inferior cerebral vein [10] and 2 mm above. Retraction was not necessary. After occlusion, the MCA was transected to avoid recanalization. The craniectomy was covered with a small piece of Gelfoam, the soft tissues were allowed to fall back into place, and the skin was sutured. Rats recovered from anesthesia on the heating pad and were returned to their cages.

Groups Studied

In the pretreatment group, kynurenic acid (300 mg/kg) was administered intraperitoneally at the same time as the MCA

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was occluded; 2 additional doses were given at 4-hour intervals (Group IA, n = 20). Saline controls (Group IB, n = 10) were employed. In the posttreatment group, kynurenic acid (300 mg/kg) was administered intraperitoneally at 1 hour after the MCA occlusion and 2 additional doses were given at 4-hour intervals (Group IIA, n = 10). Saline controls (Group IIB, n = 10) were also employed. Sham-operated controls, not treated, constituted Group III (n = 4).

Treatment Administration

Kynurenic acid (Sigma K 3375) was dissolved in a small amount of NaOH 1N on a stirring plate for 5 minutes. The solution was then diluted with 0.9% NaCl saline. The pH was adjusted to 7.4 with HCl. The injection volume of kynurenate or saline was approximately 3 ml per dose.

Neurological Examination

The neurological status of each rat was evaluated by a blinded rater 24 hours after MCA occlusion. The following grading scale was used: Grade 0, no observable deficit; Grade 1, forelimb flexion; Grade 2, forelimb flexion and decreased resistance to lateral push [10].

Neuropathological Evaluation

After neurological examination, rats were anesthetized with chloral hydrate and a lethal intracardiac perfusion of 2,3,5-triphenyltetrazolium chloride (TTC) was given [11]. This water-soluble salt is enzymatically converted in intact mitochondria to a deep red fat-soluble formazan compound [12, 13], allowing immediate quantification of infarct size. We have shown that the infarct size thus measured at 24 hours after MCA occlusion in this model is not different from that measured in hematoxylin-eosin-stained sections [11]. TTC-stained brains were removed carefully and the surface was examined for any change in vascularity in the area of the stroke to verify the occlusion and sectioning of the MCA. Brains were then fixed in 10% phosphate-buffered formalin for 24 hours. A single 2-mm TTC-stained coronal section through the maximum area of infarction was photographed using color slide film (Ektachrome, tungsten, ASA 160) for measurement of the size of infarction. Size was quantified by differential cutting and weighing techniques of traced images and was expressed as percentage of the coronal section involved [10].

Statistical Analysis

Intergroup and intragroup MABP values were analyzed using repeated measures analysis of variance. Bonferroni's method was used for the post-hoc tests when necessary. The exact probability calculation was done using the Fisher least significant difference test.

Intergroup neurological outcome scores were analyzed by the nonparametric Kruskal-Wallis test corrected for tied ranks. Intragroup values were analyzed by the nonparametric Mann-Whitney test.

Intergroup infarction size values were analyzed by analysis of variance. Intragroup infarction size values were analyzed by unpaired *t*-test. A probability of less than 0.05 was considered significant.

Results

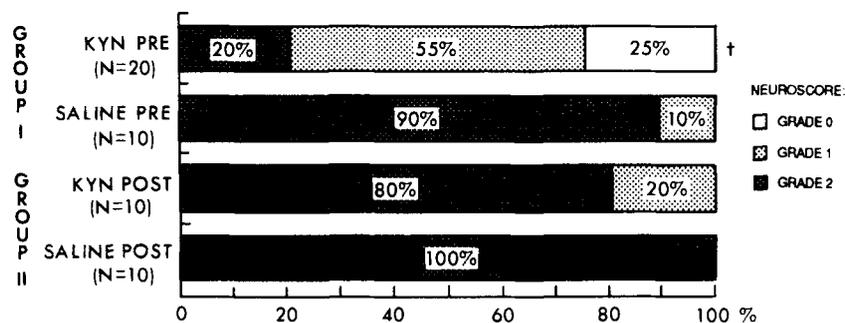
Baseline MABP values ranged from 98 ± 2 to 103 ± 4 mm Hg. The intergroup analysis of the MABP values did not show a significant change during or after treatment from pretreatment values. In addition, no difference in MABP values was found between groups (e.g., kynurenate-treated, saline-treated, or sham-operated controls).

Rats pretreated with kynurenic acid (Group IA) had a significantly better neurological outcome ($p < 0.001$) than saline controls or rats posttreated with kynurenic acid. No significant difference was found between rats posttreated with kynurenic acid (Group IIA) and controls (Group IIB) (Fig 1).

At neuropathological examination, it was found that the MCA had been occluded and transected in all operated rats; the MCA was intact in all sham-operated rats.

The size of infarction on coronal sections of rats pretreated with kynurenic acid ($17 \pm 1\%$ infarct) was significantly smaller ($p < 0.001$) than controls ($30 \pm$

Fig 1. Effects of kynurenate ($p < 0.001$, intergroup analysis) versus saline on neurological outcome (percentage of animals in each functional Grade 0–2). Neuroscore: Grade 0 (no deficit); Grade 1 (forelimb flexion); Grade 2 (forelimb flexion and decreased resistance to lateral push) [10]. KYN PRE = kynurenate pretreatment; KYN POST = kynurenate posttreatment (1 hour after middle cerebral artery occlusion); SALINE PRE = saline pretreatment (controls); SALINE POST = saline posttreatment (controls); † = $p < 0.001$, intragroup analysis.



1% infarct) (Figs 2 and 3). No significant difference in hemispheric infarction size was found between rats posttreated with kynurenic acid ($31 \pm 2\%$ infarct) and control rats ($35 \pm 1\%$ infarct) (Fig 3).

A significant linear correlation ($r = 0.861$; $p < 0.001$) was found between the size of the infarcted area and the results of the neurological examination (Fig 4).

Discussion

Excitatory amino acid neurotransmitters have been implicated in neuronal cell death in the acute injury states of ischemia [14], anoxia [15], hypoglycemia [8, 9], and prolonged seizures [5, 16], as well as in the chronic degenerative disorders Huntington's disease [17], motor neuron disease [18], and possibly Alzheimer's disease [19]. Our current data extend these observations to an experimental model more compatible with the clinical phenomenon of stroke: irreversible occlusion of the MCA. These experiments used pretreatment with kynurenate, a mixed receptor antagonist effective at both NMDA and non-NMDA excitatory receptors. The results demonstrate a 43% attenuation in infarct size (Figs 2 and 3; $p < 0.001$) and a marked attenuation in resultant motor impairment (Fig 1; $p < 0.001$) in kynurenate-treated animals.

Precisely which receptor blockade is responsible for this attenuated infarct size is uncertain because of the relatively nonspecific inhibition of excitatory neurons by kynurenate. In the spinal cord, kynurenate blocks excitation at all receptor subtypes [20], but in the hippocampus it acts preferentially on the NMDA and kainate receptors [21, 22]. The effects of kynurenate do not seem to be caused by a nonspecific suppression of cellular activity, as acetylcholine-induced excitation is unaffected in cortical cells in vivo by kynurenate administration [23]. Similar conclusions have been reached in the hippocampal slice [24] and in the cat caudate nucleus [25], in which kynurenate failed to alter resting membrane potential induced by current injections.

The NMDA receptor has received major attention for these "excitotoxic" effects. Our initial observation of the attenuation of ischemic neuronal injury using intrahippocampal injections of the specific NMDA antagonist AP-7 has been confirmed using systemic administration of the same compound [7]. Additional specific NMDA antagonists MK-801 [26] and 3-(+)-2-carboxypiperazin-4-yl propyl-1-phosphonic acid (CPP) [27] have been recently demonstrated to attenuate neuronal cell death in the hippocampus in the model of global ischemia followed by reperfusion. Using hypoxic injury in neuronal cell cultures, the specific NMDA antagonists 2-amino-5-phosphonovalerate, AP-7, and the dissociative anesthetic ketamine permit greater than 90% neuronal survival after 20 hours of hypoxia; nontreated cultures demon-

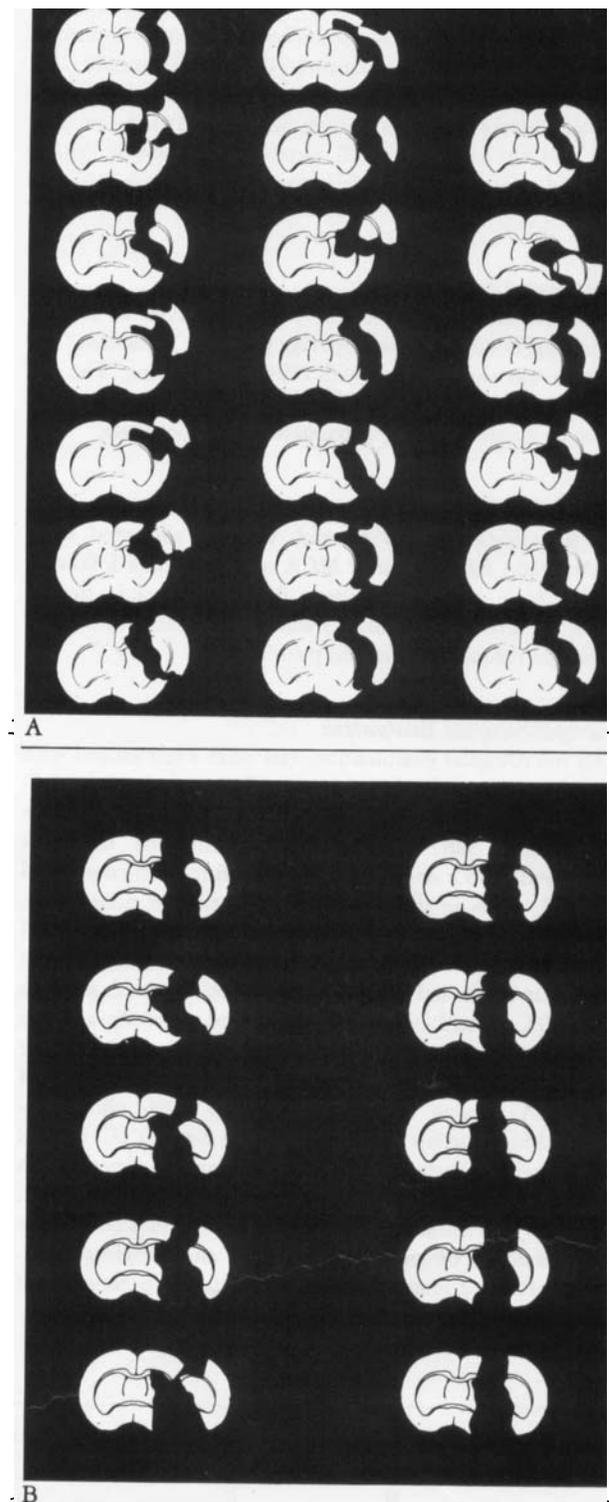


Fig 2. Pattern and size of infarction in (A) kynurenate pretreatment rats (Group IA), and (B) saline-treated rats (Group IB). Silhouettes are drawn from rat brain coronal sections of each animal. The offset portion (right side of each section) represents the infarcted area.

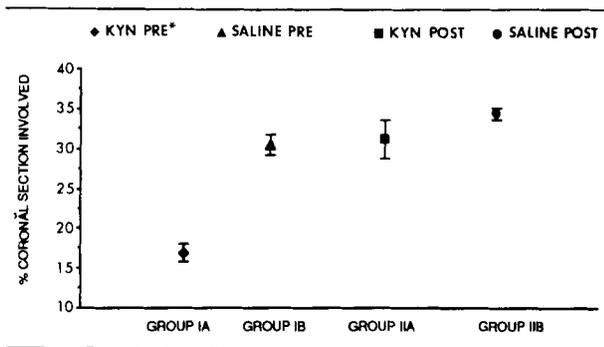
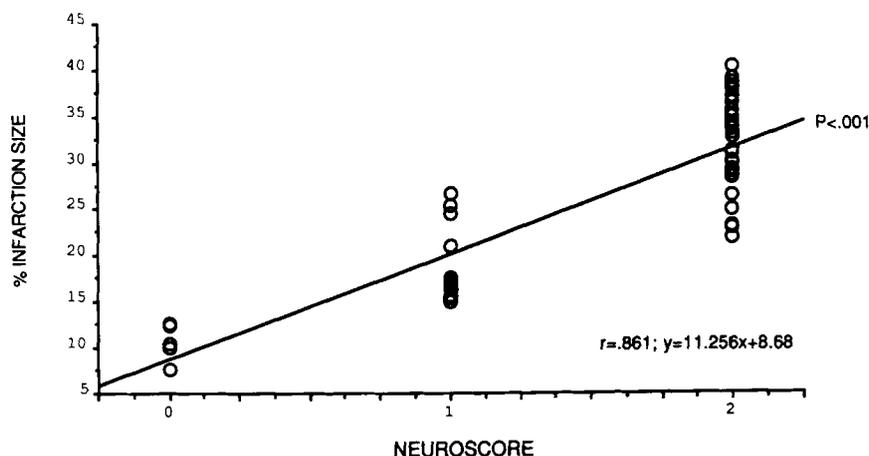


Figure 3. Effects of kynurenat on infarction size ($p < 0.001$, intergroup analysis). KYN PRE = kynurenat pretreatment; KYN POST = kynurenat posttreatment (1 hour after middle cerebral artery occlusion); SALINE PRE = saline pretreatment (controls); SALINE POST = saline posttreatment (controls). Data are expressed as means \pm SEM and represent the percentage of the coronal section infarcted. * = $p < 0.001$, intragroup analysis.

strated nearly complete cellular degeneration [28]. Similar results in neuronal culture were reported with the nonselective antagonists kynurenat and D-glutamyl glycine. However, in these studies, no protective effect was observed after administration of glutamate antagonists, which have primary effects at non-NMDA receptors: gamma-glutamylaminomethyl sulfonate or glutamate-diethylester. Further, kynurenat, but not gamma-glutamylaminomethyl sulfonate, attenuated glutamate neurotoxicity in cortical cell cultures [28]. That NMDA antagonists may affect neuropathological change resulting from excitatory amino acids in vivo is supported by the finding of rapid elevations of extracellular glutamate and aspartate in the hippocampus during ischemia in the intact rat [29]

Fig 4. Linear correlation between neurological outcome and size of infarction ($r = 0.861$; $p < 0.001$). Data for size of infarction (percent infarct size) expressed as percentage of coronal section infarcted from Figures 2 and 3. Neuroscore: Grade 0 (no deficit); Grade 1 (forelimb flexion); Grade 2 (forelimb flexion and decreased resistance to lateral push).



and the rapid release of endogenous glutamate from rat brain synaptosomes after anoxia [30].

The rationale for the use of excitatory amino acid inhibitors as a potential therapy for ischemia [31] is based on the observation of increased cell excitation in selectively vulnerable neurons after ischemia [32] and/or the very rapid firing rate of these selectively vulnerable cells [33, 34]. That such a pathophysiological mechanism may be operative in focal ischemia as well as global ischemia/reperfusion [32] is supported by the increased metabolic activity in the infarct rim after MCA occlusion [35]. In this study, the 2-deoxyglucose technique was used after MCA occlusion in rats. Decreased metabolism is seen in the lateral striatum. But this central ischemic core is surrounded by a broad area of increased metabolic activity within the cortical mantle comprising the infarct rim. Thus cell excitation after ischemia may occur in models of both focal [32] and global [35] ischemia.

In our experiments, pretreatment, but not posttreatment (1 hour after MCA occlusion), was effective in decreasing infarct area. This time-limited effect of kynurenat may be a result of the transient stability of the ischemic penumbra. In primates studied 2 weeks after MCA occlusion, only microscopic areas of infarction were seen after 15 to 30 minutes of ischemia where large infarcts were invariable after 2 hours [36]. Further, the attenuated cerebral blood flow and decreased intracellular pH remained stable for the first 3 hours after MCA branch occlusion in the rabbit, with subsequent neurochemical deterioration and resultant ischemic cell change seen at neuropathological examination [37]. These observations regarding the duration of the ischemic penumbra are compatible with the time-limited effect of kynurenat in these studies.

The experiments reported here extend the concept of cell excitation as a critical variable in neuronal cell death caused by ischemia from models of global ischemia/reperfusion [6, 7, 26, 27] to a model of permanent focal cerebral vascular occlusion. The former

model is compatible with the clinical syndrome of anoxic encephalopathy (e.g., after cardiac arrest), and the latter with the clinical syndrome of stroke. The studies reported here, as well as those of others [7, 27, 28], support the concept that pharmacological inhibition of cell excitation may be useful therapy for the treatment of acute cerebral ischemia.

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