Neuroprotective Mechanism of (+)SKF 10,047
In Vitro and in Gerbil Global Brain Ischemia

Paul G. Lysko, PhD; Tian-Li Yue, PhD; Juan-Li Gu, MD; and Giora Feuerstein, MD

Background and Purpose: The N-methyl-D-aspartate receptor is believed to mediate part of the ischemic neuronal damage caused by the excitatory amino acid glutamate. (+)SKF 10,047, the prototypic α-agonist, interacts with the N-methyl-D-aspartate receptor. Therefore, we studied the neuroprotective effect of (+)SKF 10,047 on cultured rat cerebellar neurons and on CA1 hippocampal neurons of gerbils exposed to brain ischemia.

Methods: Mechanisms of neuroprotection were studied in vitro by measuring calcium influx into cultured rat cerebellar granule cells loaded with fura 2-AM. In vivo neuroprotection of gerbil CA1 hippocampal neurons was studied in a posttreatment regimen following 5 minutes of bilateral carotid artery occlusion and 7 days of reperfusion.

Results: In primary cultured rat cerebellar granule cell neurons, (+)SKF 10,047 in a dose-dependent manner diminished intracellular calcium levels of N-methyl-D-aspartate-stimulated neurons by a maximum of 87% (n=8), with a 50% inhibitory concentration of 0.8 μM. (+)SKF 10,047 did not prevent subsequent calcium influx stimulated by kainic acid or KCl, nor did it interfere with modulation of the kainate response by quisqualic acid. Neuroprotection of 64% (p=0.006, n=15) of gerbil CA1 hippocampal neurons was achieved by posttreatment injection followed by minipump infusion.

Conclusions: Neuroprotection by (+)SKF 10,047 most likely involves interaction at the N-methyl-D-aspartate receptor. These results suggest that the benzomorphan class of α-agonists may provide neuroprotection in cerebral ischemia and stroke.

KEY WORDS • amino acids • cerebral ischemia • neuroprotection • gerbils

We have recently reported that the prototypic α-agonist SKF 10,047 (N-allylnormetazocine) as well as other benzomorphans were neuroprotective against the excitotoxic action of glutamate in our in vitro ischemia model system of cultured rat cerebellar granule cells.1,2 (+)SKF 10,047 is thought to be neuroprotective by acting as a noncompetitive antagonist at the N-methyl-D-aspartate (NMDA) receptor channel, exerting an anticonvulsant action like MK-801 by blocking cation influx initiated by the excitatory amino acids glutamate and aspartate.1-6 (+)SKF 10,047 also binds with high affinity to a haloperidol-sensitive α-site, which is better defined by the more specific α-ligands (+)-3-PPP[3-(3-hydroxyphenyl)-N-(1-propyl)piperidine] and DTG (1,3-di-o-tolylguanidine).7,8 We have recently reported the neuroprotective effects of (+)SKF 10,047 and MK-801 given as a pretreatment in the gerbil model of global brain ischemia.2 In that study, we found that at equivalently neuroprotective doses, (+)SKF 10,047 allowed five times faster recovery from motor deficits than did MK-801, exhibited far fewer hypothermic effects, and was neuroprotective in a postischemic treatment regimen. The objectives of the present study were to extend our previous research on the mechanisms of action of (+)SKF 10,047 by examining its ability to interfere with NMDA-evoked calcium mobilization in cultured neurons and examining whether (+)SKF 10,047 exerted significant neuroprotective efficacy when given after an ischemic insult. We report the efficacy of a postischemic treatment regimen that employs the use of minipump infusion of (+)SKF 10,047 to protect against CA1 hippocampal loss.

Materials and Methods
Primary cultures of rat cerebellar neurons were prepared from 8-day-old Sprague-Dawley rat pups (Taconic Farms, Inc., Germantown, N.Y.) and used after 8 or 9 days in culture.9-11 For measurements of the intracellular calcium concentration ([Ca2+]i), cells were grown on 10.5×35 mm glass coverslips (Wheaton Industries, Millville, N.J.) in 60-mm dishes (Nunc, Roskilde, Denmark), loaded with 2 μM fura 2-AM (Calbiochem Corp., La Jolla, Calif.) in buffer for 1 hour, and allowed to equilibrate at 37°C for 5 minutes in fresh buffer of the following millimolar composition: 154 NaCl, 5.6 KCl, 2.3 CaCl, 5.6 glucose, and 8.6 N-(2-hydroxyethyl) piperazine-N'-[2-ethanesulfonic acid] adjusted to pH 7.4 with NaOH. Fluorescence of fura-2 in cell monolayers immersed into 2 ml buffer in a stirred cuvette was measured as previously described,12-14 and [Ca2+]i was calculated with a few modifications. Coverslips were aligned at 45° to the light path with a custom-made,

From the Department of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.
Address for correspondence: Paul G. Lysko, PhD, Cardiovascular Pharmacology, Department of Pharmacology, PO Box 1539, L-510, King of Prussia, PA 19406-0939.
Received January 30, 1992; final revision received May 18, 1992; accepted May 26, 1992.
slotted, Plexiglas holder designed by J. Paul Hieble of this department to fit tightly inside the cuvette. \( F_{\text{max}} \) was determined by adding ionomycin to 35 \( \mu M \) and \( F_{\text{min}} \) by adding 400 mM ethylene glycol-\( \beta \)-aminoethyl ether N\(^\prime\),N\(^\prime\),N\(^\prime\),N\(^\prime\)-tetraacetic acid (EGTA) in 4M Tris (pH 10) to a final concentration of 10 mM EGTA, essentially the same as has been recently reported\(^{13,14} \); our results gave very similar values. A prior report of a procedure\(^{15} \) for determining these values in granule cell neurons caused (in our hands) a falsely high \( F_{\text{max}} \), leading to an underestimation of basal \([\text{Ca}^{2+}]_i \) levels.

The Mongolian gerbil model of global forebrain ischemic stroke has been extensively studied and characterized.\(^{16-20} \) In brief, male Mongolian gerbils weighing 60–70 g (Tumblebrook Farms, West Brookfield, Mass.) were anesthetized with 2.5% isoflurane in a mixture with 100% \( O_2 \), placed on a 37°C heating pad to keep body temperature changes to <1° during ischemia,\(^2 \) exposed to bilateral carotid artery occlusion for 5 minutes, and allowed to recover on the heating pad for 10 minutes. After 7 days, bilateral neuronal counts over a 750-\( \mu \)m section of the hippocampal CA1 regions stained in 0.1% thionin were determined by microscopic examination of coded slides to ensure unbiased observation. For protection studies, \((+)\text{SKF 10,047} \) was dissolved in saline and administered subcutaneously as a bolus and was infused with the use of minipumps. Minipumps (Alzet model 1003D, Alza Corp., Palo Alto, Calif.) were loaded with 100 \( \mu l \) \((+)\text{SKF 10,047} \), activated by immersion in normal saline for 4 hours at 37°C, and implanted subcutaneously [two per gerbil to deliver a total of 270 mg/kg \((+)\text{SKF 10,047} \) over 3 days].

The enantiomer \((+)\text{SKF 10,047} \) was obtained through the National Institute on Drug Abuse, Rockville, Md.

Data in text and figures are mean±SEM values of the indicated number of animals. Statistical analysis was performed as indicated in the figure legends; a significant difference was accepted at \( p<0.05 \).

Results

The effect of 5 minutes' ischemia is seen in Figure 1 for vehicle-treated gerbils, in which the CA1 neuronal count was decreased to an average of 27% (44/163, \( n=15 \)) of the mean count in sham-operated animals (163) 7 days after ischemia. Administering \((+)\text{SKF 10,047} \) as a bolus of 15 mg/kg at 1 and 2 hours after reperfusion and as a 90 mg/kg/day infusion for 3 days was an effective posttreatment regimen (64% protection, 104/163, \( n=15 \), \( p=0.006 \)).

In our previous investigation we never saw any evidence for \((+)\text{SKF 10,047} \)-induced neurotoxicity,\(^2 \) and here we examined the effects of \((+)\text{SKF 10,047} \) on conscious, nonischemic gerbils. There was no difference in CA1 neuronal counts when animals treated with 60 mg/kg s.c. \((+)\text{SKF 10,047} \) were compared with saline-treated control gerbils 7 days after injection (Figure 2).

Measurements of \([\text{Ca}^{2+}]_i \), in primary cultured rat cerebellar granule cells gave resting values of 145±19 nM (\( n=8 \)), similar to recently published values\(^{13,14} \) that were higher than the low levels previously reported,\(^9 \) most likely due to differences in the determination of \( F_{\text{max}} \) and \( F_{\text{min}} \) (see "Materials and Methods"). Stimulation with 5 \( \mu M \) glycine and 100 \( \mu M \) NMDA increased \([\text{Ca}^{2+}]_i \), almost threefold to 419±73 nM (\( n=8 \)) over basal levels, providing sustained or gradually increasing levels of \([\text{Ca}^{2+}]_i \) (Figure 3). \((+)\text{SKF 10,047} \) diminished the stimulated \([\text{Ca}^{2+}]_i \), by a maximum of 87±2% (\( n=8 \)), as
FIGURE 3. Tracings showing inhibition by (+)SKF 10,047 of sustained intracellular calcium concentration ([Ca$^{2+}$]$_i$) increases in rat cerebellar granule cells stimulated with N-methyl-D-aspartate (NMDA). A: After additions of 5 μM glycine (●) and 100 μM NMDA (△), (+)SKF 10,047 was sequentially added to trace 1 (downward arrows) at increasing micromolar concentrations shown. Subsequent additions included 10 μM MK-801, 100 μM kainic acid (KA), 100 μM quisqualic acid (QA), and 30 mM KCl. B: Neurons received 5 μM each of glycine and nimodipine before addition of 100 μM NMDA (△). (+)SKF 10,047 was added as in A (upward arrows, trace 2) at increasing micromolar concentrations shown, followed by same additions as in A.
The interruption of cell calcium homeostasis by excitatory amino acids is considered to be an important initiator of excitotoxic damage,21 especially in delayed neuronal death. In cerebellar granule cells, NMDA causes sustained increases in $[Ca^{2+}]_{i}$ as clearly shown in the present study. The primary influx is through the receptor-associated channel, with secondary influx through voltage-sensitive channels that are inhibited by nifedipine.13 The agonist-operated calcium channel flux can be blocked rapidly by MK-801,13,15 as we have shown here for (+)SKF 10,047. The $IC_{50}$ of 0.8 $\mu$M and the cumulative levels of (+)SKF 10,047 needed to totally block the channel (16.65 $\mu$M, Figure 4) reflect the levels we reported for protection against glutamate-induced excitotoxicity.1 The (+)SKF 10,047 effect was selective for only the NMDA subtype of glutamate receptor because, as reported for MK-801,13 subsequent stimulation of neurons with kainate caused immediate calcium influx and prior treatment with nifedipine did not alter the potency of (+)SKF 10,047. Similarly, the inhibitory response at the kainate receptor by quisqualate, which has been assessed in a variety of ways in these cells,15,22,23 persisted after the addition of (+)SKF 10,047, again supporting the selectivity of (+)SKF 10,047's action at the NMDA receptor. These results strengthen our previous suggestion that benzomorphans cross-reactivity at NMDA receptors affords neuroprotection with diminished side effects.2 Although the protective mechanism of (+)SKF 10,047 in vitro indicates efficacy at, and selectivity for, the NMDA receptor, NMDA receptor activation in vivo is not the only mechanism involved in selective neuronal vulnerability. Other factors, such as non-NMDA receptors, must be involved in neuronal death in vivo because noncompetitive inhibitors such as (+)SKF 10,047 do not provide total neuroprotection2 (Figure 1).

The results of the minipump protection study, in which treatment was delayed until 1 hour after reperfusion, reinforces our previous findings that (+)SKF 10,047 is an effective neuroprotectant even when treatment is initiated after ischemia.2 The neuroprotective effect of the minipump treatment was the same as that of our previous posttreatment regimen of 15 mg/kg at 15 minutes and 1 hour and 30 mg/kg at 2, 4, and 6 hours, which produced no significant decrease in temporalis muscle temperature until after 4 hours of treatment.2 The minipump was implanted 20 minutes after the 100-minute therapeutic window of opportunity for reversal of delayed neuronal injury with halothane, an inhibitor of postischemic hyperthermia.20,24 The neuroprotective effect of the minipump treatment regimen further supports a pharmacological effect for (+)SKF 10,047 independent from hypothermia. Drug-induced hypothermia is an important consideration in neuroprotection studies because many authors have shown that simply lowering brain temperature by only 2°C prevents neuronal damage after forebrain ischemia17-20-25-27 while others have reported the hypothermic protection afforded by MK-801.18,19 Other studies of the temperature effects of SKF 10,047 in mice and rats have shown mostly no effect, with occasional hyperthermia.28-29 However, doses of (+)SKF 10,047 higher than used here (80 mg/kg) have been reported to produce acute hypothermia in rats30 as we have reported for 60 mg/kg in gerbils.31 Because acute administration of (+)SKF 10,047 at 5-40 mg/kg s.c. produced no temperature effects in rats30 and we saw either no hypothermia or significant resolution (15-30 minutes) of hypothermia in gerbil brain,2 neuroprotection by (+)SKF 10,047 is likely to be independent of hypothermia.

We also do not envision a neuroprotective effect in vivo by increasing cerebral blood flow because (+)SKF 10,047 causes only small increases in heart rate and mean arterial blood pressure in conscious dogs32 and has no contractile effect at up to 100 $\mu$M in rat tail artery.33 In fact, (+)SKF 10,047 potentiated norepinephrine-induced contractions in rat tail artery34 and, in general, benzomorphans have been shown to constrict isolated canine cerebral vessels, but only at concentrations around 0.1-1 mM.34 Therefore, if (+)SKF 10,047
has an effect on cerebral vessels, one could expect only vasocostriction and reduced blood flow.

Although noncompetitive inhibitors of the NMDA receptor have been reported to cause neaurhopathologic changes such as vacuole formation and stimulation of heat shock protein HSP72 in rat brain, we observed no pathological changes in the hippocampal CA1 regions of gerbils receiving up to 60 mg/kg (+)SKF 10,047. Therefore, this class of noncompetitive NMDA receptor inhibitors may be better tolerated.

These results offer further evidence that the neuroprotective effects of (+)SKF 10,047 are manifested at the NMDA receptor and that this class of compound may prove useful in the treatment of cerebral ischemia and stroke.

Acknowledgments

We would like to thank Ms. Christine Webb of Cardiovascular Pharmacology for expert technical assistance and Mr. Robert C. Gagnon of Research Statistics for excellent statistical analysis of the data.

References

Neuroprotective mechanism of (+)SKF 10,047 in vitro and in gerbil global brain ischemia.
P G Lysko, T L Yue, J L Gu and G Feuerstein

Stroke. 1992;23:1319-1323
doi: 10.1161/01.STR.23.9.1319

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/23/9/1319

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/