Protective Effect of FR115427 against Ischemic Hippocampal Damage in Gerbils

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ABSTRACT—Excitatory amino acids and their receptors have been postulated to be involved in mediating ischemic neuronal damage. We occluded the bilateral carotid arteries for 5 min in gerbils to examine the effect of FR115427, a novel *N*-methyl-*D*-aspartate (NMDA) antagonist, on ischemic neuronal damage. FR115427 prevented hippocampal CA1 cell damage at a dose of 10 mg/kg and reduced spontaneous locomotor hyperactivity in gerbils after the development of ischemia at a dose of 32 mg/kg. The effective doses of MK801 were 3.2 mg/kg for preventing hippocampal CA1 cell damage and 1 mg/kg for reducing spontaneous locomotor hyperactivity. Moreover, we monitored the changes in body temperature of ischemic gerbils for 24 hr. The body temperature of ischemic gerbils significantly increased 1 hr after reperfusion. The pretreatment with FR115427 or MK801 prevented the hyperthermia provoked 1 hr after reperfusion in ischemic gerbils. In addition, the hypothermia was developed in gerbils treated with MK801 24 hr after reperfusion. However, FR115427 did not show hypothermia at any time. These results indicate that FR115427 has a protective effect against ischemic hippocampal CA1 cell damage after systemic administration, and this protective effect appears to be due to anti-NMDA activity.

Keywords: *N*-Methyl-*D*-aspartate (NMDA), Cerebral ischemia, Hippocampal damage, FR115427, MK801

It has been postulated that the brain neuronal degeneration associated with ischemia may result from excessive release of excitatory amino acids such as glutamate (1). This hypothesis has been supported by reports that *N*-methyl-*D*-aspartate (NMDA) antagonists protect the brain from ischemic damage (2–4). FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride, Fig. 1) is a novel non-competitive NMDA antagonist (5). In the present experiment, we assessed the effects of FR115427 on hippocampal delayed neuronal cell damage in the CA1 region and spontaneous locomotor hyperactivity induced by transient global ischemia in gerbils and compared these effects with those of the non-competitive NMDA antagonist MK801.

Moreover, postischemic hypothermia and hyperthermia have been reported to play an important role in ischemic hippocampal damage in gerbils (6, 7). It has been demonstrated that the protective effect of MK801 against ischemic injury in gerbils appears to be due to hypothermia (7, 8). However, the lack of protection of CA1 neurons by acute postischemic hypothermia and the lack of aggravation by acute postischemic hyperthermia have been reported (9, 10). In addition, it has been reported

that the degree of postischemic hypothermia may depend on the duration of systemic anesthesia (11). Therefore, it is interesting to monitor the changes in body temperature during ischemic insults in the absence of systemic anesthesia. Furthermore, to clarify whether FR115427 and MK801 affect body temperature, we also monitored the body temperature of ischemic and normal gerbils treated with these compounds.

MATERIALS AND METHODS

Animals

Male Mongolian gerbils (*Meriones unguiculatus*) aged 9–11 weeks (Japan SLC, Hamamatsu) were used. The animals were housed four to a cage and allowed free access to food and water, and a 12-hr light/dark cycle was maintained.

Transient cerebral ischemia in gerbils

Gerbils were restrained, and 20–30 mg of lidocaine (Fujisawa Pharmaceutical Co., Ltd., Osaka) was sprayed onto the surface of the neck and subcutaneously after incision of the skin. Under local anesthesia, both common

FR115427

MK801

Fig. 1. Chemical structures of FR115427 and MK801.

carotid arteries were exposed and clamped with micro-clips. Five minutes later, the clips were removed and reperfusion was verified. Before starting the surgery, it was confirmed by general reaction to skin pinch with forceps that gerbils did not suffer from pain. The test drugs were injected intraperitoneally 30 min before occlusion.

Measurement of locomotor activity

Twenty-four hours after ischemia and reperfusion, the locomotor activity of each animal was measured for 10 min using a BTA-1 (Muromachi Kikai Co., Ltd., Tokyo) which is a high density, optical beam animal movement monitor with 48 infrared beams crossing the monitored area (50 × 50 cm).

Histology

Four days after ischemia, the gerbils were anesthetized with thiopental (50 mg/kg, i.p.), and the brains were fixed via cardiac perfusion at a pressure of 140 cmH₂O. The fixative consisted of 1% formaldehyde in 0.1 M phosphate buffer (PH 7.4). After perfusion fixation, the brains were removed and kept in the same fixative for 3 days and then embedded in paraffin. Three-micrometer-thick coronal sections were taken between 1.5 and 2.0 mm posterior to the bregma, and then they were stained with cresyl violet. In each gerbil, the number of normal-appearing CA1 neurons per 1-mm linear length of stratum pyramidale was counted bilaterally by light microscopy at ×400 magnification according to the method of Kirino et al. (12), and the cell numbers were averaged.

Measurement of body temperature

At room temperature (25 ± 1 °C), the rectal temperature of gerbils was measured at various times under restrained condition using a Thermo-Finer (Terumo Co., Ltd., Tokyo). FR115427 (32 mg/kg) or MK801 (3.2 mg/kg) was injected intraperitoneally 30 min before ischemia.

Data analysis

Statistical analysis of locomotor activity and neuronal density were performed by the Mann-Whitney *U*-test, and ED₅₀ values of the protective effect against ischemia were estimated by the Probit method. The relationship between locomotor activity and the number of CA1 pyramidal cells was investigated by linear correlation coefficients (*r* values). The effects on body temperature were tested by Dunnett's multiple comparisons.

Drugs

FR115427 and MK801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a,d*)cyclohepten-5,10-imine) were synthesized at the New Drug Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. (Osaka). FR115427 was dissolved in saline, and MK801 was dissolved in aqueous diluted HCl and neutralized by 1 N NaOH.

RESULTS

Effects on locomotor activity after cerebral ischemia

Twenty-four hours after occlusion of the bilateral carotid arteries for 5 min, the locomotor activity of

Table 1. Effects of FR115427 and MK801 on locomotor activity and hippocampal CA1 pyramidal cell damage in gerbils after cerebral ischemia

Treatment ^a	Dose (mg/kg)	n	Locomotor activity (cm/10 min) ^b	Neuronal density (number/mm) ^b
Sham-operated		7	3027 ± 237**	201 ± 7**
Ischemia		7	9184 ± 1218	4 ± 1
FR115427	1	6	7645 ± 1397	25 ± 17
	3.2	6	7900 ± 1331	33 ± 25
	10	7	6160 ± 831	92 ± 30**
	32	5	2635 ± 993**	167 ± 41*
Sham-operated		5	4137 ± 68**	181 ± 2**
Ischemia		6	11176 ± 59	5 ± 1
MK801	0.32	6	9543 ± 1647	4 ± 1
	1	5	5634 ± 1292*	87 ± 47
	3.2	7	N.T.	107 ± 36**
	10	5	N.T.	179 ± 6**

^a Gerbils were treated with drug (i.p.) 30 min before ischemia.

^b Values are expressed as the mean ± S.E. N.T.: Not tested.

*P < 0.05; **P < 0.01 vs. ischemia group (Mann-Whitney *U*-test).

gerbils increased markedly, as compared with the sham-operated animals. FR115427 significantly reduced the postischemic hyperactivity at a dose of 32 mg/kg. MK801 (1 mg/kg) also significantly reduced hyperactivity after cerebral ischemia (Table 1). However, since ataxia was observed in gerbils treated with MK801 (3.2, 10 mg/kg) even 24 hr after ischemia, locomotor activity was not measured at these doses.

Effects on hippocampal CA1 pyramidal cell damage induced by cerebral ischemia

Four days after transient cerebral ischemia, the number of neurons per millimeter of the CA1 region was reduced markedly, as compared with that of the sham-operated group. FR115427 ameliorated ischemia-induced neuronal cell damage dose-dependently at an ED_{50} of approximately 9.9 mg/kg and significantly attenuated the neuronal cell damage at doses as large as 10 mg/kg. MK801 also ameliorated the neuronal cell damage dose-dependently at an ED_{50} of 1.6 mg/kg and significantly prevented the cell damage at doses as large as 3.2 mg/kg (Table 1). Representative photographs of a hippocampal CA1 neuron are shown in Fig. 2.

The relationship between locomotor activity and hippocampal CA1 pyramidal cell damage

The statistically significant correlations between locomotor activity and the CA1 pyramidal cell number were as follows: FR115427-treated ischemic gerbils ($r = -0.808$, $P < 0.01$) and MK801-treated ischemic gerbils ($r = -0.740$, $P < 0.01$).

Effects on body temperature after ischemia

There was no difference between the body temperature of the normal and sham-operated animals. In the ischemic group, hypothermia was observed 5 min after reperfusion, and an increase in body temperature was observed 1 hr after reperfusion, but the body temperature at 3 and 24 hr after reperfusion did not differ from that of the sham-operated animals (Table 2). FR115427 (32 mg/kg) and MK801 (3.2 mg/kg) significantly inhibited hyperthermia 1 hr after reperfusion. In addition, MK801 produced hypothermia 24 hr after reperfusion. On the other hand, hypothermia was not observed in gerbils treated with FR115427 (Table 2).

Effects on body temperature in normal animals

Animals treated with FR115427 (32 mg/kg) were normothermic at 0.5, 1.5, 3.5 and 24 hr after administration (Table 3). However, treatment with MK801 (3.2 mg/kg) produced hypothermia at 24 hr after administration.

Fig. 2. Representative photographs of hippocampal CA1 neurons of stratum pyramidale 4 days after occlusion of the bilateral carotid artery in gerbils for 5 min. Cresyl violet staining. Upper: Sham operation. The CA1 pyramidal cells were well-preserved. Middle: Ischemia. Note marked damage to the CA1 pyramidal cells. Lower: FR115427 treatment (32 mg/kg). Most of the CA1 pyramidal cells were preserved. Magnification $\times 400$.

DISCUSSION

Transient global cerebral ischemia produces delayed neuronal death in gerbils (13). The hippocampal CA1 and CA2 pyramidal neurons are most vulnerable to cerebral ischemia, and they are rich in NMDA receptors (14). In addition to these facts, it has been shown that various

Table 2. Effects of FR115427 and MK801 on body temperature in gerbils after cerebral ischemia

Treatment ^a	Dose (mg/kg)	n	Immediately before occlusion	Immediately after occlusion	After reperfusion			
					5 min	1 hr	3 hr	24 hr
Normal	—	7	38.1 ± 0.1 ^b		38.0 ± 0.1**	38.5 ± 0.1**	38.4 ± 0.1	38.3 ± 0.1
Sham-operated	—	7	38.2 ± 0.1		38.0 ± 0.1**	38.4 ± 0.1**	38.2 ± 0.1	38.3 ± 0.1
Ischemia	—	7	38.0 ± 0.2	37.4 ± 0.2	36.9 ± 0.2	39.6 ± 0.1	38.4 ± 0.1	38.1 ± 0.1
FR115427	32	6	37.7 ± 0.1	37.1 ± 0.2	36.6 ± 0.2	38.7 ± 0.2**	38.2 ± 0.1	38.3 ± 0.1
MK801	3.2	7	37.4 ± 0.3	36.7 ± 0.3	36.3 ± 0.3	38.6 ± 0.2**	38.4 ± 0.2	36.1 ± 0.3**

^a Gerbils were treated with drug (i.p.) 30 min before occlusion. ^b Values are expressed as the mean ± S.E. (°C). **P < 0.01 vs. ischemia group (Dunnett's multiple comparisons).

Table 3. Effects of FR115427 and MK801 on body temperature in normal gerbils

Treatment	Dose (mg/kg)	n	Before administration	After administration			
				30 min	1.5 hr	3.5 hr	24 hr
Normal	—	5	38.4 ± 0.1 ^a	38.8 ± 0.1	38.8 ± 0.2	38.5 ± 0.2	38.6 ± 0.2
FR115427	32	5	38.6 ± 0.1	38.9 ± 0.2	38.8 ± 0.1	38.8 ± 0.1	38.4 ± 0.2
MK801	3.2	5	38.5 ± 0.1	38.8 ± 0.3	38.7 ± 0.1	38.0 ± 0.1	37.7 ± 0.2*

^a Values are expressed as the mean ± S.E. (°C). *P < 0.05 vs. normal group (Dunnett's multiple comparisons).

NMDA antagonists reduce ischemia-induced neuronal damage. For example, competitive NMDA antagonists, 2-amino-7-phosphonoheptanoic acid (AP7), 4-(3-phosphonopropyl)-2-piperazine-carboxylic acid (CPP) and *cis*-4-(phosphonomethyl)-2-piperidine-carboxylic acid (CGS19755), protect against hippocampal damage after transient cerebral ischemia in gerbils (2, 3). In the present experiment, FR115427 and MK801 prevented hippocampal damage after cerebral ischemia, and these effects were dose-dependent. The doses of FR115427 and MK801 that produced 50% protection were 9.9 and 1.6 mg/kg, respectively. FR115427 was sixfold less potent than MK801. It has been reported that FR115427 and MK801 have affinity for the NMDA receptor/ion channel (5). FR115427 inhibited the binding of (H³)MK801, and the binding affinity of FR115427 was fourfold less potent in the absence of L-glutamate and 14-fold less potent in the presence of L-glutamate than those of MK801 (5). Furthermore, electrophysiological experiment with a cortical wedge preparation demonstrated that the blockade of NMDA-mediated responses by FR115427 was non-competitive (5). The *in vitro* NMDA-antagonist effect of these compounds almost parallel their activity in preventing hippocampal CA1 damage.

We monitored the spontaneous changes in body temperature of ischemic gerbils in the absence of systemic anesthesia. Ischemic animals developed hypothermia immediately after reperfusion and became hyperthermic 1 hr af-

ter reperfusion and returned to the normothermia 3 hr after reperfusion. Hypothermia has been demonstrated to prevent ischemic neuronal death in many animals such as gerbils (8) and rats (6). In addition, it has also been reported that hypothermia reduces the release of extracellular glutamate in ischemic animals. However, the present experiment demonstrates that the hippocampal neuronal damage that occurred though hypothermia was developed immediately after reperfusion. It has been suggested that the mild reduction of 1.1 °C observed in the present experiment was not enough to protect against the neuronal damage.

In the present experiment, the body temperature of ischemic gerbils increased to 39.6 °C 1 hr after reperfusion. It has been reported that hyperthermia exacerbates ischemia-induced neuronal damage and elevation of the cerebral temperature to 39 °C results in complete destruction of neurons in the CA1 region (15).

Pretreatment with FR115427 or MK801 inhibited this hyperthermia, but the body temperature of the treated animals was not lower than that of the sham-operated animals. Further experiments will be necessary to clarify whether hyperthermia at 1 hr in the absence of systemic anesthesia is the major aggravating factor and whether the protective effect of these compounds is based on their ability to prevent post ischemic hyperthermia.

On the other hand, the body temperature of ischemic animals treated with MK801 significantly decreased 24 hr

after ischemia as compared with the sham-operated animals. Moreover, normal animals given MK801 were hypothermic 24 hr after injection, although FR115427 did not induce hypothermia at any measured time. The blocking action of MK801 on NMDA responses was persistent, with only partial recovery after a washing period of 3 hr in electrophysiological studies using a cortical slice preparation (16). If the hypothermia induced by MK801 is due to NMDA antagonism, persistent blockade of NMDA responses may produce hypothermia at 24 hr after injection.

At present, it is not clear why hyperthermia is induced by reperfusion after ischemia. It is possible that post-ischemic hyperthermia results from the changes in the levels of thermoactive substances such as glutamate, serotonin and prostaglandins. It has been demonstrated that glutamate release increases during and after ischemia in gerbils (17). The injection of glutamate into the hypothalamus led to dose-dependent hyperthermia at doses between 10 and 40 $\mu\text{g}/\text{rat}$ (18).

In the present experiments, the non-competitive NMDA antagonists FR115427 and MK801 are effective in reducing ischemia induced locomotor hyperactivity in gerbils, and this protective effect is related to the degree of hippocampal damage. Similar data on the competitive NMDA antagonist CGS19755 was observed (data not shown). Therefore, measurement of postischemic behavioral changes may be useful for evaluating the degree of hippocampal CA1 damage.

In summary, FR115427, a non-competitive NMDA antagonist, has a protective effect against ischemic damage in global cerebral ischemic gerbils in the absence of systemic anesthesia. It is supposed that these protective effects may be entirely the results of the blockade of NMDA receptors. The effect is dose-dependent, and the difference of potency of the protective effects between FR115427 and MK801 are almost comparable with the *in vitro* (^3H)MK801 binding results.

Furthermore, we monitored the body temperature of the ischemic gerbils in the absence of systemic anesthesia and observed spontaneous changes in temperature after ischemia. Hypothermia after ischemia and hyperthermia 1 hr after reperfusion were observed. Moreover, FR115427 and MK801 prevented the hyperthermia. Therefore, it is suggested that this hyperthermia may be glutamate-dependent.

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