Cerebroprotective Effects of a Novel Pyrazoline Derivative, MS-153, on Focal Ischemia in Rats

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Received July 10, 1996 Accepted January 20, 1997

ABSTRACT—MS-153 ((R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline) is a novel pyrazoline compound that has potent cerebroprotective effects in the rat focal cerebral ischemia model. Middle cerebral artery (MCA) occlusion in rats allows detailed assessment of both functional and morphological sequelae of brain infarct. Using this model, we evaluated the cerebroprotective effects of MS-153. Treatment with MS-153 (12.5 mg/kg, i.v. bolus followed by 6.25 mg/kg/hr or 25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr infusion for 7 days) significantly reduced infarct volumes and improved neurological deficits in MCA occluded rats 7 days after occlusion. Delayed treatment significantly reduced infarct volume 24 hr after MCA occlusion when MS-153 (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr infusion for 21 hr) administration was started 3 hr after occlusion. Brain edema was also significantly improved when MS-153 (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr infusion for 18 hr) administration was started 6 hr after occlusion.

Keywords: MS-153, Middle cerebral artery occlusion, Focal cerebral ischemia, Cerebroprotection, Therapeutic window

Ischemic stroke is one of the most common cerebrovascular disorders and causes major disability to survivors. The major pathophysiological mechanism of ischemic tissue damage can be explained by the glutamate-calcium (Ca2+) hypothesis (1). In in vitro experiments using cultured neurons, glutamate is cytotoxic (2). In in vivo experiments using models of cerebral ischemia, massive amounts of excitatory amino acids (glutamate and aspartate) are released into the extracellular space during cerebral ischemia (3–6). This massive accumulation of extracellular glutamate leads to excessive accumulation of intracellular Ca2+ via activation of glutamate receptors (7, 8) and voltage-sensitive Ca2+ channels (9). The uncontrolled elevation in intracellular Ca2+ leads to neuronal cell death (10). Sustained glutamate accumulation has been also observed in a stroke patient (11). Thus excitatory amino acids play a crucial role in the development of ischemic tissue damage; and suppression of glutamate release (12, 13), blocking of glutamate receptors (14, 15) or antagonism of various Ca2+ channels (16–18) can provide therapeutic interven-

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stroke. To evaluate the usefulness of a drug, it is essential to determine a therapeutic window, a period of time following stroke when a drug can reduce damage and improve outcome (14, 23). A drug is more useful if it has a wider therapeutic window.

MATERIALS AND METHODS

A model of focal ischemia

Male Sprague-Dawley rats (Japan SLC, Hamamatsu), 9-weeks-old, weighing 250–350 g were used. Rats were anesthetized with a mixture of halothane and oxygen-nitrogen (30% oxygen and 70% nitrogen). Two to three percent halothane was used to induce anesthesia and 1–1.5% was used to maintain anesthesia during the operation. The left femoral vein was cannulated with a polyethylene catheter (Intramedic PE-10, PE-50; Becton Dickinson & Company, Parsippany, NJ, USA), passed subcutaneously under the back and out the dorsal neck. The catheter was connected to an infusion pump (SP-10; Nipro, Osaka) through a cannula swivel (375/22; Insteck, Plymouth Meeting, PA, USA), and MS-153 or saline was administered through the catheter. For the permanent occlusion model: the left MCA was occluded according to Tamura et al. (21) with a slight modification. The rats underwent subtemporal subperiosteal craniectomy (with intact zygoma). The left MCA was exposed, and the MCA trunk was permanently electro-cauterized from its origin including the lenticulostriate branches to its junction with the inferior cerebral vein. Body temperature was maintained at 37.0–37.5°C with a heat lamp during the surgical procedure. After the operation, to connect the catheter, rodent jackets (Alice King, Los Angeles, CA, USA) were put on the rats, which were then kept in individual breeding cages.

Infarct volume and neurological deficits 7 days after MCA occlusion

Since MS-153 has a very short half life in serum when injected as an i.v. bolus, i.v. infusion was selected as the administration route to evaluate the efficacy of MS-153 in the rat MCA occlusion model. Two separate experiments were conducted according to the dose. In either experiment, the administration of MS-153 was started immediately after MCA occlusion and continued for 7 days. In the low dose experiment, the rats were divided into three groups and given either saline (N=9) or MS-153 as a 3.13 mg/kg, i.v. bolus followed by i.v. infusion of 1.56 mg/kg/hr for 7 days (N=8) or MS-153 as a 6.25 mg/kg, i.v. bolus followed by i.v. infusion of 3.13 mg/kg/hr for 7 days (N=8). In the high dose experiment, the rats were given either saline (N=10) or MS-153 as a 12.5 mg/kg, i.v. bolus followed by i.v. infusion at 6.25 mg/kg/hr for 7 days (N=10) or MS-153 as a 25 mg/kg, i.v. bolus followed by i.v. infusion at 12.5 mg/kg/hr for 7 days (N=10). Seven days after MCA occlusion, neurological deficits were evaluated by the degree of hemiparesis (24) and forelimb flexion (25). Three features were independently observed for the assessment of hemiparesis: walking ability, paresis of the hind legs and muscle power of the forelimbs. Hemiparesis was assessed as the summed score of the three items. Forelimb flexion was assessed by holding the rat by the tail one meter above the floor and observing the position of the front limbs. These neurological deficits were graded using the criteria listed in

| Table 1. Neurological deficits grading |

| I. Hemiparesis | Score |
|----------------|-------|
| (1) walking ability | |
| circulate | 1 |
| stagger | 2 |
| walk normally | 3 |
| (2) paresis of the hind leg | |
| drag the hind legs | 1 |
| noticeable difference between right and left legs | 2 |
| no difference between right and left legs | 3 |
| (3) muscle power of the forelimbs | |
| weak | 1 |
| normal | 2 |
| II. Forelimb flexion | |
| severe abduction of right shoulder | 1 |
| mild abduction of right shoulder | 2 |
| extend both forelimbs | 3 |
Table 1. Grading was conducted in a blind manner.

After neurological deficits were evaluated, the brains were fixed by perfusion with 3.5% formaldehyde in 0.1 M phosphate buffer (pH 7.4) under pentobarbital anesthesia and stored in the same solution for 7 days. Six coronal sections (2-mm-thick) were cut from the frontal pole. Six thin slices (5 μm) were cut from the sections and stained by the Klüver-Barrera method. The slides were made and projected to scale, and the surface area of necrotic tissue was determined in a blind manner with an image analyzer (IMM-256V8; Mitani Shouzu, Fukui). The infarct volume was calculated from the surface area of necrotic tissue measured at 2.0-mm intervals.

Delayed treatment

Delayed treatment with MS-153 was conducted according to Hatfield et al. (14), Smith et al. (26) and Graham et al. (27). The rats were divided into four (infarction study) or six (edema study) groups with MS-153 administered at different times following MCA occlusion. Rats were given either saline or MS-153 (25 mg/kg, i.v. bolus followed by i.v. infusion at 12.5 mg/kg/hr for the specified number of hours). Twenty-four hours after occlusion, the rats were sacrificed under pentobarbital anesthesia, and brain water contents and infarct volumes were determined using different rats for each measurement.

In the infarction study, the administration of MS-153 was started immediately, 3 or 6 hr after MCA occlusion and continued until sacrifice. Twenty-four hours after MCA occlusion, the animals were decapitated under pentobarbital anesthesia and the brains were rapidly removed. Six coronal sections (2-mm-thick) were cut from the frontal pole and incubated for 20 min at 37°C in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline (28). The stained sections were stored in 3.5% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr. The sections were photographed to scale, and the surface area of necrotic tissue was determined in a blind manner with an image analyzer (IMM-256V8, Mitani Shouzu). The infarct volume was calculated from the surface area of necrotic tissue measured at 2.0-mm intervals.

Statistical analyses

The Dunnett's test followed by ANOVA or Dunnett's type of non-parametric multiple comparison test followed by the Kruskal-Wallis test was used to determine the statistical significance of the differences in infarct volume, grade of neurological deficits, and brain water content. Numerical data are each expressed as a mean ± S.D.

RESULTS

Effects on infarct volume and neurological deficits

None of the 55 rats died within 7 days of MCA occlusion. There was a consistent pattern of ischemic brain damage after permanent MCA occlusion. Consistent with an earlier report (21), lesions were observed in the cortex of the frontal lobe and the lateral part of the caudate nucleus. The total infarct volumes of the low dose treatment with MS-153 were 121±26 mm³ (3.13 mg/kg, i.v. bolus followed by 1.56 mg/kg/hr for 7 days) and 122±45 mm³ (6.25 mg/kg, i.v. bolus followed by 3.13 mg/kg/hr for 7 days), which are not significantly different from the infarct volume of the control group (vehicle-treated) (134±33 mm³). In the high-dose treatment group (Fig. 2), treatment with a 12.5 mg/kg, i.v. bolus followed by 6.25 mg/kg/hr for 7 days significantly reduced the total infarct volume by 49% (from 125±53 mm³ in the control (vehicle-treated) rats to 64±23 mm³ in the MS-153-treated rats; P<0.01). At a higher dose (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr for 7 days), MS-153 produced a more profound reduction of 54% in infarct volume (from 125±53 mm³ in the control (vehicle-treated) rats to 58±18 mm³ in the MS-153-treated rats, P<0.001). All control (vehicle-treated) rats showed distinct neurological deficits.
except walking ability 7 days after MCA occlusion (Table 1). Focal Ischemia induced by MCA occlusion did not cause any abnormalities in walking ability, and no significant difference in walking ability between control (vehicle-treated) and MS-153-treated rats was observed. In the low-dose group, treatment with MS-153 at either 3.13 mg/kg, i.v. bolus followed by i.v. infusion of 1.56 mg/kg/hr for 7 days or 6.25 mg/kg, i.v. bolus followed by i.v. infusion of 3.13 mg/kg/hr for 7 days did not improve any behavioral abnormalities in MCA-occlusion rats (data not shown). In the high dose group (Fig. 3), treatment with MS-153 (12.5 mg/kg, i.v. bolus followed by 6.25 mg/kg/hr) significantly improved abnormalities in both paresis of the hind legs and muscle power of the forelimbs. At a higher dose (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr), MS-153 significantly improved abnormalities in forelimb flexion as well as abnormalities in paresis of the hind legs and muscle power of the forelimbs. Total neurological scores were plotted against infarct volumes to examine the correlation between neurological deficits and infarct volume (Fig. 4). A linear correlation between total neurological score and infarct volume was observed with a correlation coefficient of 0.711 (N=30, P<0.01).

Delayed treatment with MS-153

Infarction study: Figure 5 shows the changes in infarct volume according to time. The infarct volume quickly increased in the first several hours, and the increase in infarct volume continued until 24 hr after MCA occlusion. As shown in Fig. 6, when administration was started immediately after MCA occlusion, treatment with MS-153 (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr) resulted in a significant reduction in infarct volume of 37% (from 179±48 mm³ in the control rats to 113±39 mm³ in the MS-153-treated rats, P<0.01). A 3-hr delay in the start of MS-153 treatment also produced a significant reduction in infarct volume of 27% (from 179±48 mm³ in the control rats to 130±44 mm³ in the MS-153-treated rats, P<0.05). However, no significant reduction in infarct volume was observed when administration was started 6 hr after MCA occlusion (from 179±48 mm³ in the control rats to 152±36 mm³).

Brain edema formation study: Changes in brain edema formation are also shown in Fig. 5. The increase in brain water content due to edema formation was rapid in the first several hours but not as fast as the increase in infarct volume, and the increase in brain water content continued until 24 hr after MCA occlusion. As shown in Fig. 7, 24 hr after MCA occlusion, the brain water content in the ipsilateral hemisphere of the control (vehicle treated) rats was significantly greater than in that in the contralateral hemisphere (from 78.7±0.20% in the contralateral to 81.8±0.70% in the ipsilateral hemisphere, P<0.001), showing formation of ischemic brain edema. Delayed treatment with MS-153 (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr) produced a significant reduction in brain water content in the ipsilateral hemisphere.
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Fig. 4. Scatterplot of the relationship between infarct volume and total neurological scores 7 days after permanent middle cerebral artery occlusion in rats. ○: Control group, △: MS-153 group treated with 12.5 mg/kg, i.v. bolus followed by 6.25 mg/kg/hr for 7 days and ×: MS-153 group treated with 25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr infusion for 7 days. The scatterplot gives a correlation coefficient of 0.711 (N=30, P<0.01).

Fig. 5. Time course of percent changes in infarct volume and brain edema after middle cerebral artery occlusion in rats. MCA-occluded animals were sacrificed at the indicated times after occlusion. Coronal sections were made and stained with hematoxylin-eosin. Infarct volumes and water contents were measured as described in Materials and Methods. Brain edema was calculated from the changes in water content between the left hemisphere and right hemisphere. Values at 24 hr after MCA occlusion were considered as 100%, and other data are expressed as percentages relative to the 24-hr values. □: percent brain edema, ○: percent infarct volume.

Fig. 6. Effect of delayed MS-153 administration on infarct volume 24 hr after middle cerebral artery occlusion. Administration of MS-153 (25.0 mg/kg, i.v. bolus followed by 12.5 mg/kg/hr) was started immediately, 3 or 6 hr after middle cerebral artery occlusion and continued until sacrifice (24 hr after middle cerebral artery occlusion). Values are the mean±S.D. of the infarct volumes for ten animals. Significant reductions in infarct volume are *P<0.05 and **P<0.01 compared to the control group.

DISCUSSION

Infarction and edema formation are two main pathophysiological changes observed in cerebral ischemia. Severe focal ischemia results in coagulation necrosis in the ischemic zone, which leads to well-demarcated infarction. Morphometric analysis of infarct volume is commonly employed to determine the efficacy of cerebroprotective agents in preclinical trials. Neurological

81.8±0.7% in the control rats to 81.1±0.6% (immediate treatment, P<0.01), 81.0±0.5% (1-hr-delayed treatment, P<0.01), 80.8±0.4% (3-hr-delayed treatment, P<0.001) and 81.0±0.4% (6-hr-delayed treatment, P<0.01). Twelve-hours-delayed treatment with MS-153 gave no significant reduction in the water content in the ipsilateral hemisphere (from 81.8±0.7% in the control rats to 81.3±0.6% in the rats given the 12-hr-delayed
deficits provide another index to assess the extent of ischemic brain injury following focal cerebral ischemia. Ischemic lesions of the frontal cortex and striatum of rats often lead to neurological deficits, including hemiparesis, and the severity of neurological deficits has been correlated with an increase in the size of the lesion (25, 29). Our results clearly demonstrate that MS-153 reduces the size of the 7-day infarct volume and improves neurological deficits in MCA-occluded rats when administration is started immediately after occlusion. Significant correlation between infarct volume and neurological deficits in this study indicates that improved neurological outcome is due to reduction of infarct volume induced by MS-153 treatment (Fig. 4). These results imply that MS-153 is cerebroprotective when applied to a model of focal ischemia.

In the delayed treatment with MS-153, the effects of MS-153 on infarct volume and brain edema of MCA-occluded rats were determined 24 hr after MCA occlusion. In this experiment, infarct volumes (179 ± 48 mm³) in control rats were relatively larger than the 7-day infarct volumes (134 ± 33 mm³). In the early stages of focal ischemia in rats, infarct formation is always accompanied by swelling of the ischemic tissue (brain edema), and swelling of ischemic tissue due to brain edema often results in an enlargement of the infarct zone (30). In the rat permanent MCA occlusion model, brain edema subsides 7 days after MCA occlusion (31) and the infarct volume measured 7 days after MCA occlusion is relatively smaller than the 24-hr infarct volume. Two different methods were employed to determine infarct volumes in the experiments. In the delayed treatment experiments, brain tissues were stained with TTC without prior fixation with formaldehyde. On the other hand, in the 7-days treatment study, the tissues were fixed by perfusion with 3.5% formaldehyde in 0.1 M phosphate buffer before staining, and the tissues were dehydrated with the fixing fluid. Dehydrated tissues give slightly smaller measurements of infarct volume than non-dehydrated tissues. Thus brain edema and the non-dehydrating staining method (TTC staining) would give relatively large infarct volumes in the early stages of ischemia. However, the formation of infarction is irreversible and very rapid in the early several hours after the onset of ischemia and as shown in Fig. 5, MS-153 reduces infarct volume in rats when administration is started within 3 hr after MCA occlusion. MS-153 also reduces brain edema in rats when administration is started within 6 hr after MCA occlusion. As shown in Fig. 5, infarct volume rapidly increases by 6 hr after MCA occlusion, and after that point, it gradually increases until 24 hr after occlusion. In brain edema formation, the brain water content also quickly increases by 12 hr after occlusion and gradually increases until 24 hr after onset on ischemia. Figure 5 implies that the first several hours is the critical point to prevent development of ischemic damage. It is quite interesting that MS-153 still prevents development of infarction when it is administered 3 hr after onset of ischemia. MK-801, a well-known N-methyl-D-aspartate receptor antagonist, is reported to reduce infarct volume only when administered within 1 hr after the onset of ischemia (14, 23). It is also reported that the typical AMPA antagonist NBQX reduces infarct volume when administered 90 or 60 min (32) after MCA occlusion, but that it is not effective when administered 120 min after MCA occlusion (33). The novel sodium channel blocker BW619C89 is
also reported to reduce infarct volume when it is administered 1 hr after onset of ischemia, but not cerebroprotective on 2-hr-delayed treatment (26). Thus, MS-153 has one of the widest therapeutic windows achieved by a cerebroprotective agent in permanent focal ischemia in rats.

It has been observed in several microdialysis studies that glutamate levels in the cerebral extracellular space are increased during ischemia. In permanent cerebral ischemia, the glutamate level in the cerebral cortex is gradually increased, and this high level of glutamate is maintained during ischemia (5, 6). Since MS-153 suppresses extracellular glutamate accumulation in the ischemic penumbra zone during permanent occlusion of the middle cerebral artery in rats (19, 20), the observed cerebroprotective effects must be relevant to suppression of accumulation of glutamate in the extracellular space. Thus these results suggest that MS-153 provides a promising therapeutic intervention for acute stroke.

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