Effect of a New Calcium Entry Blocker, NB-818, on Delayed Neuronal Death in the Ischemic Gerbil Hippocampus

Kazuo Kamei, Sonoko Tsuchida, Kazuhiro Taguchi and Masaru Nishikibe*

Central Research Laboratories, Banyu Pharmaceutical Co., Ltd.,
2-9-3 Shimomeguro, Meguro-ku, Tokyo 153, Japan

Received November 15, 1990 Accepted March 22, 1991

ABSTRACT—The effect of NB-818, a new dihydropyridine calcium entry blocker, on delayed neuronal death (DND) in the hippocampal CA1 subfield of gerbils after 5 minutes of forebrain ischemia induced by bilateral carotid artery occlusion was examined. Gerbils were treated intraperitoneally with NB-818 (0.1–3 mg/kg) just after release of the occlusion. Four days after the ischemia, they were fixed by perfusing 10% buffered-formalin, and the neuronal cell density (NCD, cell/mm) in the CA1 subfield was estimated under microscopy. The average NCD in the ischemic control group was 43 ± 10.8 cells/mm, whereas NB-818 (3 mg/kg) significantly ameliorated DND with an average NCD of 143 ± 24.2 cells/mm (P < 0.01). In addition, NB-818 (3 mg/kg) significantly inhibited DND at 1, 2 and 4 weeks after transient ischemia: the average NCD of the NB-818 and ischemic control groups were 80 ± 9.4 (P < 0.01) and 43 ± 7.7 cells/mm, 92 ± 13.7 (P < 0.05) and 52 ± 9.3 cells/mm, and 57 ± 5.0 (P < 0.01) and 43 ± 12.4 cells/mm, respectively. In this experiment, NB-818 exhibited a protective effect on DND in the hippocampal CA1 subfield after transient forebrain ischemia, and its effect persisted for up to 4 weeks. These findings suggest that NB-818 may be useful for clinical treatment of neurological deficit after an ischemic insult.

After short term transient cerebral ischemia, the neurons in certain specific areas can be injured. This phenomenon is called selective vulnerability (1, 2). In the hippocampus, morphological changes in the CA1 pyramidal cells can be detected by light microscopy at least 2 days after brief ischemia, followed by an extensive loss of CA1 pyramidal cells at 4 days. This slow process of cell death is known as delayed neuronal death (DND), which has been found by Kirino using Mongolian gerbils (3, 4). This phenomenon has been also observed in rats (5, 6) and humans (7). Recently, it has been proposed that excitatory neurotransmitters serve as the mechanism by which this selective and delayed pathogenesis occurs in neurons (8): the excitatory amino acid glutamate (Glu) exists at the presynaptic level and is released excessively during ischemia and/or immediately after recirculation. The released Glu may activate the N-methyl-D-aspartate (NMDA) subtype of the Glu receptor, which is followed by an increase in Ca\(^{2+}\) conductance in membranes and promotion of excessive Ca\(^{2+}\) inflow into neuronal cells, culminating in cell death. It has been recognized that...
NMDA receptors exist in the hippocampal CA1 subfield at a very high density (9, 10). The ability of NMDA antagonists (11, 12) or calcium entry blockers (13–16) to prevent DND has been examined using ischemic models with hippocampal CA1 neuronal damage in gerbils or rats, to assess whether or not these drugs ameliorate the neuronal damage. Although there are many reports showing the efficacy of drugs against DND, all of them have reported histological assessments of animals sacrificed up to seven days after ischemia, and a protective effect of drugs on DND over a long period after ischemia has not yet been observed.

NB-818, isopropyl methyl 2-carbamoyloxy-methyl-6-methyl-4-(2,3-dichlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate, has already been reported to have a remarkable vasodilative effect in isolated cerebral vessels (17), and it produces a selective and long-lasting increase in the cerebral blood flow (18–20). In addition, we have found that NB-818 improves the decreased cerebral blood flow (21) and reduces the mortality rate in the gerbil ischemic model (22). In the present experiment, we examined the effects of NB-818 on DND of ischemic hippocampal CA1 neurons in gerbils during an observation period of 4 weeks after transient brief ischemia.

MATERIALS AND METHODS

Procedures to induce ischemia

Male Mongolian gerbils, weighing 50–80 g, purchased from Seiwa Experimental Animals Co., were used in this experiment. They were anesthetized with 4% halothane, placed on their backs, and anesthesia was maintained with 1.5–2.0% halothane. After a ventral midline cervical incision was made, the right and left common carotid arteries were exposed and freed from the vagus nerves carefully, and then nylon threads were put around each artery to make placing or removing aneurysm clips easy. Ischemia was induced by occlusion of both common carotid arteries using clips. Anesthesia was discontinued just before the clips were placed. After 5 minutes of carotid occlusion, the clips and the threads were removed, allowing recirculation of the blood, and then the incised skin was closed with a surgical adhesive agent. After the operation, the animals were kept in warmed cages on heating blankets (37°C). When their righting reflexes were recovered, they were returned to their cages and allowed free access to food and water. If the gerbils did not show disappearance of the righting reflex during the carotid artery occlusion or soon after its removal, they were excluded from this experiment, because the forebrain ischemia might have been insufficient.

Histology

After the scheduled interval following ischemia, the gerbils were sacrificed by transcardiac perfusion-fixation. They were anesthetized with pentobarbital-Na (60 mg/kg, i.p.) and fixed by intracardiac perfusing of 10% buffered-formalin at a pressure of 130 cmH2O for 10 minutes. Their brains were then removed from the calvariums, immersed in the same fixative for several days, and then cut into small blocks. The plane of the coronal section was selected to include the hippocampal area 0.5–1.0 mm posterior to the most rostral tip of the hippocampus or 1.4–1.9 mm posterior to the bregma. The specimens were dehydrated through a graded series of ethanol solutions, soaked in xylene and then embedded in paraffin. Six series of 5-μm sections were prepared, and three series were stained with cresyl fast violet; the remainder was kept as a reserve sample. After the total linear length of the CA1 sector was measured by a microscope with a scale attached to the eye lens at a magnification of 100×, the number of surviving pyramidal cells in the CA1 subfield was counted at a magnification of 200×, and then the neuronal cell density (NCD, the number of CA1 neurons per 1 mm linear length of the stratum pyramidale observed in each 5-mm section) was calculated. The average of both hemispheres in three preparations was regarded as the NCD value of the indi-
individual animal. The procedure was conducted in a blind fashion throughout the microscopic observation.

Drug treatment

Experiment 1: NB-818 (0.1, 0.3, 1 or 3 mg/kg) or pentobarbital-Na (40 mg/kg) were administered intraperitoneally just after recirculation. MK-801 was administered intraperitoneally at the dose of 1 mg/kg at one hour before carotid occlusion followed by 3 mg/kg at 30 minutes after recirculation. For the ischemic control group, the animals were injected intraperitoneally just after recirculation with the vehicle (10% polyethylene glycol 300 and 10% ethanol in physiological saline) used as the solvent for NB-818. Non-ischemic untreated gerbils were kept as a normal control group. Four days after transient ischemia, the animals were fixed by perfusing 10% buffered formalin.

Experiment 2: NB-818 (3 mg/kg) or vehicle was administered intraperitoneally just after the recirculation. At 1, 2 or 4 weeks after ischemia, the animals were sacrificed, and the protective effect of NB-818 was examined.

The following drugs were used: NB-818 (synthesized by Banyu Pharmaceutical Co.), pentobarbital-Na (Somnopentyl, Pitman-Moore Inc.), and MK-801 (obtained from Merck Sharp and Dohme International, U.S.A.). The dose of MK-801 is expressed in terms of the base because the maleate salt form (conversion factor 1.52) was used. NB-818 was dissolved in the vehicle. Pentobarbital-Na and MK-801 were diluted or dissolved in physiological saline.

Statistics

The NCD of each group is expressed as the mean ± S.E.M. Significance of the difference between each drug treatment group and the ischemic group was assessed using the Mann-Whitney U-test (P < 0.05).

RESULTS

Experiment 1

Figure 1 illustrates the histopathology of the CA1 region of the hippocampus in the sham-operated, vehicle-treated and NB-818-treated animals. Gerbils treated with 3 mg/kg, i.p. of NB-818 just after the end of occlusion showed protection from neuronal cell death in the CA1 region of the hippocampus 4 days after the 5-minute occlusion. The average NCD of each group sacrificed 4 days after transient ischemia is shown in Fig. 2. The NCD in the normal control group was 269 ± 6.8 cells/mm, and all the animals showed more than 200 cells/mm. On the other hand, the NCD in the ischemic control group was 43 ± 10.8 cells/mm, and all the animals except for one showed less than 50 cells/mm. In the NB-818 treated groups, the NCD was 33 ± 4.9 cells/mm for 0.1 mg/kg, 48 ± 15.5 cells/mm for 0.3 mg/kg, 42 ± 9.1 cells/mm for 1 mg/kg, and 143 ± 24.2 cells/mm for 3 mg/kg. The NCD of animals treated with NB-818 at the dose of 3 mg/kg was significantly (P < 0.01) higher than that of the ischemic control group. In the pentobarbital-Na and MK-801 treated groups, the NCD was 178 ± 15.4 cells/mm and 91 ± 24.7 cells/mm, respectively. These values were significantly (P < 0.01 and P < 0.05, respectively) higher than those in the ischemic control groups.

Experiment 2

The NCD of each group sacrificed 1, 2 or 4 weeks after transient ischemia is shown in Fig. 3. The NCD of the ischemic control and NB-818 (3 mg/kg) treated groups 1 or 2 weeks after transient ischemia was 43 ± 7.7 cells/mm and 80 ± 9.4 cells/mm (P < 0.01) and 52 ± 9.3 cells/mm and 92 ± 13.7 cells/mm (P < 0.05), respectively; and the values in the NB-818 treated groups were larger than those in the ischemic control groups, and the difference was statistically significant. Four weeks after transient ischemia, the NCD (57 ± 5.0 cells/mm, P < 0.01) of the NB-818 treated group was significantly higher than that of the ischemic control group (43 ± 12.4 cells/mm), but it was less than that in the groups sacrificed after 1 and 2 weeks.
Fig. 1. Histopathological alteration of the neurons in the CA1 region of gerbil hippocampus 4 days after a 5-minute occlusion. A. Sham-operated group; B. Vehicle-treated group; C. NB-818, 3 mg/kg, i.p.-treated group.
Fig. 2. Effects of NB-818, pentobarbital-Na and MK-801 on neuronal cell density (NCD) in the gerbil hippocampal CA1 subfield 4 days after a 5-minute bilateral occlusion of the carotid arteries. *P < 0.05, **P < 0.01, statistically significant difference from the vehicle-treated group.

Fig. 3. Effect of NB-818 on neuronal cell density (NCD) in the gerbil hippocampal CA1 subfield one, two and four weeks after a 5-minute occlusion. *P < 0.05, **P < 0.01, statistically significant difference from the vehicle-treated group.
DISCUSSION

Cerebral ischemia can be caused by cardiac arrest, cerebral vasospasm, etc. It is known that in the case of mild and transient ischemia, the damage occurs in certain specific neurons. Such a non-homogeneous distribution of neuronal damage is called selective vulnerability. The hippocampal CA1 subfield, zones 3, 5 and 6 in the cerebral cortex, Purkinje cells in the cerebellum and the dorsal-lateral caudate putamen are known as vulnerable areas (1, 2). Especially concerning the hippocampal CA1 subfield, many reports have been released regarding both clinical cases (1, 7, 23) and pre-clinical experiments (3–5).

Various types of drugs have been assessed so far using animal ischemic models, and some efficacy has been demonstrated. Studies with the following drugs have been reported: agents with sedative action (24–26), NMDA antagonists (11, 12) and calcium entry blockers (13–16). In particular, pentobarbital (26) and MK-801 (12) have shown clear beneficial effects in the experimental ischemic models, and these data were consistent with our present experimental data. However, studies of calcium entry blockers have yielded conflicting results (14): for example, it has been reported that nicardipine but not nimodipine showed a protective effect against the neuronal cell death in the CA1 region of the hippocampus after transient ischemia (15). Therefore, there is a possibility that DHP receptors might, at least in part, be closely related to DND in the hippocampal CA1 neurons in animals subjected to transient forebrain ischemia.

In the present study, the protective effect of NB-818, a new DHP calcium entry blocker which has a potent vasodilator effect on cerebral vessels (17), on DND in the hippocampal CA1 neurons of gerbils was observed during 4 weeks after transient ischemia. Calcium ion has been postulated as a final common denominator of ischemic cell damage. We have already reported that NB-818 ameliorated the decreased cerebral blood flow and brain edema in the gerbil unilateral carotid occlusion model (21), and it reduced the mortality rate in the gerbil bilateral carotid occlusion model (22). In the animals sacrificed 4 days after transient ischemia, NB-818 at 3 mg/kg, i.p., but not 1 mg/kg, i.p. showed a marked preventive effect against the DND of hippocampal CA1 neurons. Furthermore, when the sacrifice period was lengthened to 1, 2 or 4 weeks after ischemia, NB-818 at 3 mg/kg, i.p. also significantly inhibited DND at each period. In our previous studies, the effective dose of NB-818 was 0.01 to 0.1 mg/kg, i.p., but in this study, a higher dose (3 mg/kg) was needed to produce the protective effect against the DND in hippocampal CA1 neurons. Therefore, it is unlikely that its protective effect depends on only the increase in blood flow, because at the dose of 0.1 mg/kg, i.p., NB-818 significantly improved the reduced cerebral blood flow after ischemia in gerbils (21).

The findings that treatment with NB-818 just after the release of the occlusion was effective for a longer period suggest that it will be important to start treatment with this drug as soon as possible after transient ischemia occurs. Although NCD in the NB-818 treated group sacrificed 4 weeks after ischemia was significantly higher than that in the ischemic control group, its effect was less potent than that in the 1- or 2-week treated group. Regarding this result, the following presumptions can be considered: 1) One treatment with NB-818 may not be enough to protect against the development of DND; and 2) During the longer observation period, the excessive neuronal excitation caused by spontaneous convulsive seizures may easily affect the pyramidal cells in the hippocampus. Gerbils are generally known to be liable to have acute seizures provoked by external stimuli. Once the pyramidal cells in the hippocampus are subject to damage, they become more sensitive to extrinsic stimulation. However, this assumption cannot be concluded from only the present experiment, so further investigation is needed.

In conclusion, NB-818, a DHP calcium entry blocker, exhibited a protective effect against DND in the hippocampal CA1 subfield
after transient forebrain ischemia throughout a 4-week-observation period. These findings suggest that NB-818 may be useful for the clinical treatment of neurological deficit after ischemic insult.

REFERENCES

1 Brierley, J.B. and Graham, D.I.: Hypoxia and vascular disorders of the central nervous system. In Greenfield's Neuropathology, 4th edn., Edited by Adams, J.H., Corsellis, J.A.N., Duchen, L.W. and Edward, A., p. 125–207, Yiley, London (1984)

2 Hossmann, K.A.: Post-ischemic resuscitation of the brain: Selective vulnerability versus global resistance. Prog. Brain Res. 63, 3–17 (1985)

3 Kirino, T.: Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 239, 57–69 (1982)

4 Kirino, T. and Sano, K.: Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol. (Berlin) 62, 201–208 (1984)

5 Pulvinetelli, W.A., Brierley, J.B. and Plum, F.: Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann. Neurol. 11, 491–498 (1982)

6 Kirino, T., Tamura, A. and Sano, K.: Delayed neuronal death in the rat hippocampus following transient forebrain ischemia. Acta Neuropathol. (Berlin) 64, 139–147 (1984)

7 Petitto, C.K., Feldmann, E., Pulvinetelli, W.A. and Plum, F.: Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37, 1281–1286 (1987)

8 Cotman, C.W. and Iversen, L.L.: Excitatory amino acids in the brain focus on NMDA receptors. Trends Neurosci. 10, 263–265 (1987)

9 Monaghan, D.T., Holts, V.R., Toy, D.W. and Cotman, C.W.: Anatomical distributions of four pharmacologically distinct ^3^H-L-glutamate binding sites. Nature 306, 176–179 (1983)

10 Cotman, C.W., Monaghan, D.T., Ottersen, O.P. and Storm-Mathisjen, J.: Anatomical organization of excitatory amino acid receptors and their pathways. Trends Neurosci. 10, 273–280 (1987)

11 Simon, R.P., Swan, J.H., Griffiths, T. and Mel- drum, B.S.: Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science 226, 850–852 (1984)

12 Gill, R., Foster, A.C. and Woodruff, G.N.: Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J. Neurosci. 7, 3343–3349 (1987)

13 Deshpande, J.K. and Wieloch, T.: Amelioration of ischemic brain damage by postischemic treatment with flunarizine. Neurol. Res. 7, 27–29 (1985)

14 Vibulsresth, S., Dietrich, W.D., Busto, R. and Ginsberg, M.D.: Failure of nimodipine to prevent ischemic neuronal damage in rats. Stroke 18, 210–216 (1987)

15 Alps, B.J., Calder, C., Hass, W.K. and Wilson, A.D.: Comparative protective effects of nicardipine, flunarizine, lidoflazine and nimodipine against ischemic injury in the hippocampus of the Mongolian gerbil. Br. J. Pharmacol. 93, 877–883 (1988)

16 Yoshidomi, M., Hayashi, T., Abe, K. and Kogure, K.: Effects of a new calcium channel blocker KB-2796, on protein synthesis of the CA1 pyramidal cell and delayed neuronal death following transient forebrain ischemia. J. Neurochem. 53, 1589–1594 (1989)

17 Nishikibe, M., Fukuroda, T. and Nakajima, A.: Vasodilatory properties in vascular smooth muscle of the calcium antagonist NB-818 compared to nicardipine and nimodipine. Arch. Int. Pharmacody. Ther. 297, 98–111 (1989)

18 Nishikibe, M., Nagura, J., Kamei, K. and Fukuroda, T.: Hemodynamic effects of a dihydropyridine calcium antagonist, NB-818 (NPK-1886) in anesthetized dogs. Arch. Int. Pharmacody. Ther. 291, 7–20 (1988)

19 Nishikibe, M. and Nakajima, A.: Effects of the calcium antagonist NB-818 on cerebral blood flow. Life Sci. 43, 1715–1723 (1988)

20 Nishikibe, M., Kamei, K., Nagura, J. and Suzuki, K.: Effect of the newly synthesized calcium antagonist isopropyl methyl 2-carbamoyloxymethyl-6-methyl-4-(2,3-dichlorophenyl)-1,4-di hydropyridine-3,5-dicarboxylate on cerebral venous outflow in dogs. Arzneimittelforschung 39, 678–681 (1989)

21 Nishikibe, M.: Effect of the calcium entry blocker NB-818 on cerebral blood flow after unilateral carotid occlusion model in the Mongolian gerbil. J. Pharmacol. Exp. Ther. 246, 719–725 (1988)

22 Kamei, K., Tsuchida, S. and Nishikibe, M.: Effects of NB-818 on a transient bilateral common carotid arteries occlusion model in Mongolian gerbils. Folia Pharmacol. Japon. 93, 341–347 (1989) (Abs. in English)

23 Zola-Morgan, S., Squire, L.R. and Amaral, D.G.: Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J.
Neurosci. 6, 2950–2967 (1986)

24 Cullen, J.P., Aldrete, J.A., Jankovsky, L. and Romo-Salas, F.: Protective action of phenytoin in cerebral ischemia. Anesth. Analg. 58, 165–169 (1979)

25 Hallmayer, J., Hossmann, K.-A. and Mies, G.: Low dose of barbiturates for prevention of hippocampal lesions after brief ischemic episodes. Acta Neuropathol. (Berlin) 68, 27–31 (1985)

26 Kirino, T., Tamura, A. and Sano, K.: A reversible type of neuronal injury following ischemia in the gerbil hippocampus. Stroke 17, 455–459 (1986)