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Introduction

Experimental brain ischemia models have been widely utilized for the investigation of the mechanisms of ischemia induced brain damages and for the evaluation of a new drug. There are two different types of brain ischemia models, one is a global ischemia model which has been interested in the delayed neuronal death in the CA 1 neurons of the hippocampus[1,2], the other is a focal ischemia model induced by the unilateral middle cerebral artery (MCA) occlusion. 2-Methyl-α-Ergocryptine (CBM36-733), a new ergot alkaloid drug, has centrally dopaminergic, serotonergic and cholinergic actions[3,4] and is anticipated to improve cerebral function. In the present study, we studied the effect of CBM36-733 on ischemia-induced brain damages using both global ischemia and focal ischemia models.

Materials and Methods

1. Effect of CBM36-733 on the delayed neuronal death

A total of 52 adult male Mongolian gerbils (Meriones unguiculatus) weighing 50-70 g (14-15 weeks) were allowed free access to food and water before and after all procedures. The animals were anesthetized with a gas mixture of 70% N_2O and 2% halothane (the balance being O_2). The carotid arteries were exposed bilaterally at the neck and were separated from the surrounding tissue. Then the anesthesia was discontinued and both carotid arteries were occluded with aneurysmal clips (Sugita clip). This produced uniform forebrain ischemia[5,6]. Immediately after 5 min of occlusion, recirculation was achieved by removing the clips and the animals were injected intraperitoneally with 3 mg/kg (n = 6) or 10 mg/kg (n = 14) of CBM36-733 dissolved in 0.2% methyl cellulose solution. The non-drug treated-animals (n=15) were injected with 0.2% methyl cellulose solution as a vehicle group, and another group with 40 mg/kg of pentobarbital (n = 10) to serve as a positive control. After the operation, the animals were kept on a warm mat (39 °C) until they began to move again. Seven days after ischemia, all of the above animals and 6 control animals, not subjected to carotid ligation, were anesthetized with 50 mg/kg of pentobarbital (Nembutal), and were fixed transcardially with 3.5% formaldehyde solution at 120 cm H_2O for 20 min. After

leaving the fixed animals for 60 min at room temperature, the whole brains were removed and each brain was dissected into 7 mm coronal sections which contained the hippocampal area, and further fixation was performed for 48 hours. After fixation, the brain sections were dehydrated with graded ethanol, passed through chloroform and embedded in paraffin. Sections which were 5 μm thick and which included the dorsal hippocampi located 1.5 mm posterior to the bragma were prepared by microtome and stained with cresyl violet. The number of pyramidal cells in each CA 1 region was counted under light microscopy at a magnification of × 400. Each section of this area was photographed at a magnification of × 40, its CA 1 length was measured and the number of CA 1 neurons per mm was calculated. Only the neurons which had well-preserved perikarya and nuclei were calculated. The mean values of the CA 1 pyramidal neurons per mm bilaterally was termed neuronal density and the values were tabulated. Statistical analysis was carried out using Mann-Whitney's U-test.

2. Effect of CBM36-733 on focal ischemic brain damages

A total of 24 adult male Wistar rats of the SPF strain weighing 280-300 g were allowed free access to food and water before and after procedures. A detailed description of the surgical preparation has been previously reported[7]. In brief, after induction of anesthesia with a gas mixture of 70% N_2O and 2% halothane (the balance being O_2), the right middle cerebral artery (MCA) was occluded with a silicone rubber cylinder attached to a nylon surgical thread introduced from the bifurcation of the internal carotid artery immediately after ligation of the ipsilateral common and external carotid arteries. The cylinder was made of 4-0 nylon surgical thread (Nitcho Kogyo Co., Ltd.), 16 mm long. This cylinder was coated with silicone (Xantopren, Bayer Dental) and mixed with a hardener (Elastomer Activator) to increase the thickness of the distal 5 mm to 0.25-0.30 mm. After introducing the embolus, the internal carotid artery was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the internal carotid artery to the proximal portion of the anterior cerebral artery (ACA). The origin of the right MCA and that of right posterior communicating artery were occluded by the silicone rubber cylinder. The surgery was performed within 15 min with no bleeding. Body temperature was kept at normal limits with a heating pad. Following surgery, anesthesia was discontinued and all rats exhibited neurologic deficits characterized by left hemiparesis with upper extremity dominancy and right Horner's syndrome. After 90 min of MCA occlusion, recirculation was achieved by pulling the thread out of the internal carotid artery under the same anesthetic conditions as during surgery and the animals were injected intraperitoneally with 3 mg/kg of CBM36-733 (n=12) dissolved in 0.2% methyl cellulose solution. Non-drug treated-animals were injected with 0.2% methyl cellulose solution as a vehicle group (n = 12). Once again, the rats were allowed free access to food and water until the next procedure. Although the ipsilateral common and external carotid arteries had been ligated, the ischemic area could be reperfused

via the cerebral arterial circle (circle of Willis) through the contralateral carotid and basilar arteries, and by collateral circulation of the cortical branches of the cerebral arteries.

Intraperitoneal injection of 3 mg/kg of CBM36-733 was continued every day for 14 days. After the treatment, regional cerebral blood flow (rCBF) and cerebral metabolic rate of glucose (CMRGlc) were measured for the evaluation of the effect of CBM36-733 on focal ischemic brain damages. Two weeks after 90 min of MCA occlusion, tracheotomy was performed and the animals were ventilated. Pancronium bromide (0.6 mg/kg i.p.) was administered, and both femoral arteries and vein were cannulated to allow continuous monitoring of mean arterial blood pressure, the repeated sampling of arterial blood, and the administration of fluid. After surgical preparation of the rats, 2% halothane administration was discontinued and the rats were ventilated with a mixture of 70% N_2O and 30% O_2 allowing normoxia and normocapnia. Immediately prior to measurement of CBF and CMRGlc, blood glucose level, PaO_2, $PaCO_2$, and pH were determined within normal limits. CBF and CMRGlc were measured by the [^{14}C]iodoantipyrine (Amersham) and 2-[^{14}C]deoxyglucose (Amersham) quantitative autoradiographic methods developed by Sakurada et al[8] and by Sokoloff et al[9],respectively. Cerebral [^{14}C] tissue concentrations of the autoradiograms were determined using [^{14}C]microscale standard (Amersham) by means of a computer-based microdensitometer system (Chromoscan). Data regarding rCBF and CMRGlc were analyzed using a t-test with $p < 0.05$ and $p < 0.01$ considered to be statistically significant.

Results

1. Effect of CBM36-733 on the delayed neuronal death

The stratum pyramidale in the CA 1 region of the control group was receptive to staining as were the other CA regions in that group. Almost all cells in the CA 1 region were well-preserved under high magnification . The CA 1 region of the vehicle group was less receptive to staining. Most of the cells had disappeared, and shrunken, deeply stained cell somata, lysed nuclei and plasma membranes were observed. Many glial cells also appeared in this region. When the animals were treated with 3 mg/kg CBM36-733, most of cells were disappeared as was the same findings in the vehicle group. When the animals were treated with 10 mg/kg of CBM36-733, however, the stratum pyramidale in the CA 1 region was very receptive to staining as was the same region in the control. The contour of the plasma membrane and the nucleus of each neuron was well-preserved. In the pentobarbital group, some damaged neurons were observed in the CA 1 region, but more than 70% of neurons seemed to be intact. These findings were summarized in Fig. 1.

Differences in the neuronal densities between the control group, 10 mg/kg of CBM36-733 treated group, and pentobarbital group were not statistically significant. In short, 10 mg/kg of CBM36-733 had a potent effect on cellular protection (Table 1). More than 80% of neurons were destroyed by ischemic insult in the vehicle and 3 mg/kg of CBM36-733 treated

groups, and this deterioration was significantly greater than those of the control, 10 mg/kg of CBM36-733 and pentobarbital treated groups.

2. Effect of CBM36-733 on focal ischemic brain damages

The values of rCBF of the rats measured 2 weeks of recirculation after 90 min of MCA occlusion are shown in Table 2. In this model, the anterior neocortex (FrPaSS) and lateral part of the caudate putamen (CPu-L), which were supplied by the occluded MCA, were the regions most frequently damaged. There were no significant differences between the vehicle group and the treated group. The values of CMRGlc of the rats measured 2 weeks of recirculation after 90 min of MCA occlusion are shown in Table 3. There were no significant differences between the vehicle group and the treated group.

Discussion

The incidence of this delayed type of neuronal death was prevented by the systemic administration of 10 mg/kg of CBM36-733. The other types of drugs, a glutamate antagonist, a calcium channel blocker, co-dergocrine mesylate, and a sedative were also evaluated to be effective the delayed neuronal death using the same global ischemia model[10-12]. The chemical structure of CBM36-733 is almost analogous to that of co-dergocrine mesylate, and then its pharmacological actions are considered to be equal to co-dergocrine mesylate that these actions are associated with some neurotransmitters[11].

During ischemia, an over abundance of the neurotransmitters noradrenaline, glutamate, and dopamine are released pathologically from the axon terminals and perhaps other transmitters also released[13-15]. Pathologically enhanced release of excitatory neurotransmitters, such as noradrenaline and glutamate, increase the activity of certain types of calcium channels in the hippocampal neurons which are postsynaptic elements[16,17]. When this reaction occurs within a physiological limit, intracellular second messengers are activated and signal transmission in the cytosol for such effects as long term potentiation occurs[18,19]. In the case of ischemia, however, neurotransmitters are in excess and this induces a large influx of calcium ions into postsynaptic elements[20]. Proteolytic and lipolytic reactions are activated abnormally by excessive calcium ions and this lead to cell death[21]. So, it is logical to administer the anti-glutamatergic agent, calcium blocker or anti-noradrenergic drugs, such as co-dergocrine mesylate and its derivatives to protect this type of delayed neuronal death.

In the present study, there may be a need to deal with such factors in the prevention of delayed neuronal death in the hippocampus, but its mechanisms are still controversial. The administration of 3 mg/kg of CBM36-733 was less potent to prevent the delayed neuronal death and to improve cerebral blood flow and glucose metabolism using a focal ischemia model. A further detailed study is required in order to clarify the mechanisms of anti-ischemic actions of CBM36-733.

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Table 1. Neuronal densities in the CA 1 region 7 days after 5 min of ischemia.

		neuronal density
Control	(n=7)	69.7 ± 7.2
Vehicle (0.2% MC)	(n=15)	12.2 ± 4.4
3 mg/kg CBM36-733	(n=6)	11.6 ± 5.1
10 mg/kg CBM36-733	(n=14)	42.2 ± 8.4 *
pentobarbital 40 mg/kg	(n=10)	67.9 ± 10.8 *

Values are given in mean ± SEM (/mm). n, number of animals.
*, $p < 0.01$ as compared to vehicle group (Mann-Whitney's U-test).
MC, methyl cellulose solution.

Table 2. Regional cerebral blood flow measured after 90 minutes of middle cerebral artery occlusion followed by 2 weeks of recirculation.

Structure	Vehicle	CBM treated
Ischemic side		
FrPaM	1.36 ± 0.14	1.54 ± 0.07
FrPaSS	0.55 ± 0.08	0.50 ± 0.09
CPu(L)	0.99 ± 0.17	0.76 ± 0.11
CPu(M)	1.62 ± 0.10	1.41 ± 0.17
Hippocampus	0.90 ± 0.09	0.84 ± 0.09
Thalamus (VPN)	1.47 ± 0.14	1.60 ± 0.25
Amygdala	0.51 ± 0.08	0.61 ± 0.10
Substantia nigra	0.98 ± 0.16	1.02 ± 0.17
Pons	1.24 ± 0.12	1.31 ± 0.08
Non-ischemic side		
FrPaM	1.42 ± 0.09	1.63 ± 0.13
FrPaSS	1.63 ± 0.23	1.58 ± 0.18
CPu(L)	1.78 ± 0.05	1.59 ± 0.25
CPu(M)	1.77 ± 0.07	1.53 ± 0.25
Hippocampus	0.94 ± 0.12	0.91 ± 0.07
Thalamus (VPN)	1.70 ± 0.11	1.64 ± 0.21
Amygdala	1.33 ± 0.19	1.21 ± 0.09
Substantia nigra	0.96 ± 0.08	1.02 ± 0.04
Pons	1.39 ± 0.08	1.46 ± 0.10

Values are given in mean ± SD ml/g/min. Six animals.
FrPaM, frontoparietal cortex, motor area, supplied by anterior cerebral artery; FrPaSS, frontoparietal cortex, somatosensory area, supplied by middle cerebral artery; CPu(L), lateral segment of caudate putamen; CPu(M), medial segment of caudate putamen; Thalamus (VPN), ventral posterior nucleus of thalamus.

Table 3. Local cerebral glucose utilization measured after 90 minutes of middle cerebral artery occlusion followed by 2 weeks of recirculation.

Structure	Vehicle	CBM treated
Ischemic side		
FrPaM	67.56 ± 6.56	64.17 ± 8.00
FrPaSS	57.39 ± 7.17	56.39 ± 2.41
CPu(L)	61.83 ± 7.83	59.17 ± 8.39
CPu(M)	60.61 ± 9.28	59.72 ± 9.83
Hippocampus	63.00 ± 9.39	64.39 ± 6.56
Thalamus (VPN)	59.78 ± 4.65	65.67 ± 9.39
Amygdala	48.17 ± 9.11	51.11 ± 9.39
Substantia nigra	57.56 ± 5.61	60.33 ± 5.06
Non-ischemic side		
FrPaM	64.94 ± 8.44	67.89 ± 7.44
FrPaSS	65.67 ± 7.78	64.61 ± 7.72
CPu(L)	74.28 ± 7.89	70.33 ± 7.89
CPu(M)	74.44 ± 9.61	73.06 ± 9.94
Hippocampus	62.72 ± 7.00	67.67 ± 8.44
Thalamus (VPN)	71.61 ± 7.61	72.44 ± 9.11
Amygdala	59.17 ± 8.61	54.50 ± 9.00
Substantia nigra	45.94 ± 6.17	49.72 ± 3.44

Values are given in mean ± SD umol/100g/min. Six animals.
FrPaM, frontoparietal cortex, motor area, supplied by anterior cerebral artery; FrPaSS, frontoparietal cortex, somatosensory area, supplied by middle cerebral artery; CPu(L), lateral segment of caudate putamen; CPu(M), medial segment of caudate putamen; Thalamus (VPN), ventral posterior nucleus of thalamus.

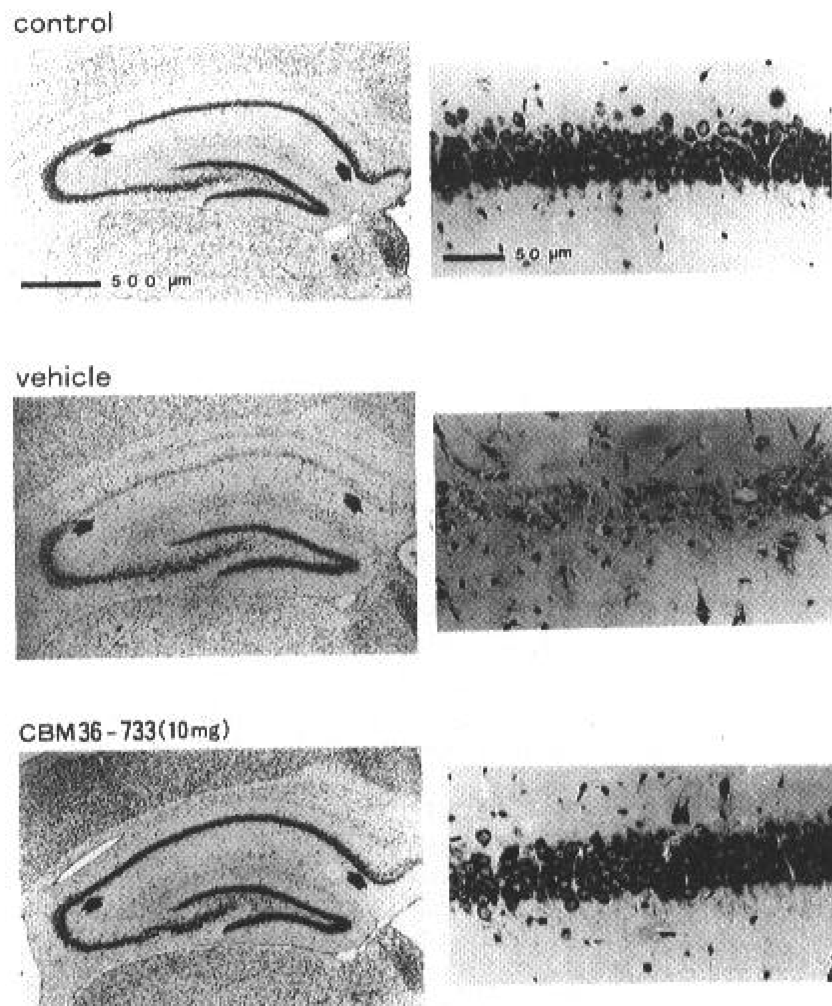

Fig. 1. Top: Coronal section of the dorsal hippocampus in the control group. The stratum pyramidale in the CA 1 region (between the arrows) is well-stained as are the other regions (left). Every CA 1 cells are well-preserved under high magunification (right). Scale bars represent 500 μm (left) and 50 μm (right).Middle: Specimen of the vehicle group. The CA 1 region is less receptive to staining (left). Most of the CA 1 neurons disappear and are replaced by glial cells (right).Bottom: Specimen of 10 mg/kg CBM36-733 treated group. The CA 1 region is receptive to staining as that of control (left). Most of the CA1 neurons are preserved within normal limits (right).