Histological Evidence for Neuroprotective Action of Nebracetam on Ischemic Neuronal Injury in the Hippocampus of Stroke-Prone Spontaneously Hypertensive Rats

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Received October 11, 1994 Accepted November 15, 1994

ABSTRACT—The protective effect of nebracetam on ischemic neuronal damage was histologically examined in the pyramidal cell layer of the hippocampal CA1 subfield 7 days after operation using stroke-prone spontaneously hypertensive rats (SHRSP) subjected to 10-min bilateral carotid occlusion. Nebracetam (50 and 100 mg/kg), given orally 10 min after reperfusion, dose-dependently protected against ischemic delayed neuronal damage in the SHRSP with occlusion; however, the blood pressure remained unchanged following nebracetam administration. These findings further support the notion that nebracetam protects against ischemic delayed neuronal cell death in the hippocampus.

Keywords: Nebracetam, Ischemic hippocampal damage, Stroke-prone spontaneously hypertensive rat

Stroke-prone spontaneously hypertensive rats (SHRSP) are a genetically determined hypertensive strain derived from spontaneously hypertensive rats (1). When these animals are fed a high-salt diet, severe hypertension occurred and their life span was short because of end-organ damage (1). The SHRSP are useful for evaluating the potency and therapeutic value of antihypertensive agents (2, 3). We demonstrated that 10-min bilateral carotid occlusion in SHRSP gradually decreased the density of the pyramidal cells in the hippocampal CA1 subfield and neuronal cell death reached a peak 7 days after the operation (4, 5). This two-vessel occlusion model of SHRSP has advantages over the four-vessel occlusion model of normal rats (6) because it avoids the coagulation of bilateral vertebral arteries. This point leads to the different dynamics of cerebral blood flow after reperfusion between our model and the four-vessel occlusion model. In this context, our model more closely simulated the post-ischemic conditions in humans. Therefore, the SHRSP with two-vessel occlusion is useful for examining delayed neuronal cell death in the hippocampus caused by a transient forebrain ischemia (4, 5). Several lines of evidence have shown that nebracetam has a nootropic property (7-12). This compound protected against hypoglycemia/hypoxia-induced striatal and hippocampal damages in vitro (8-10) and decreased the disruption of spatial cognition and memory impairment evoked by various treatments in vivo (11, 12). The present study histologically examined the protective effects of nebracetam against ischemic hippocampal neuronal damage using the two-vessel occlusion model of SHRSP.

Male SHRSP maintained and bred at the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine were used. These animals were fed a high-salt and low-protein diet containing 0.8% NaCl and 20.8% of protein (Funabashi Farm Co., Chiba) and allowed water ad libitum. Three to four rats per group were housed in a cage in an air-conditioned room at 24±1℃, humidity of 65±5%, with a 12-hr light-dark schedule (lights on 7:00 a.m.). Twelve- to fourteen-week-old SHRSP, each weighing 260-290 g, were supplied for bilateral carotid occlusion. Nebracetam was dissolved in distilled water and administered orally in a volume of 0.1 ml/100 g body weight. The control group was given distilled water as a vehicle.

The two-vessel occlusion model of the SHRSP was prepared, as described elsewhere (4, 5). In brief, the SHRSP, under halothane anesthesia (1.5% in room air), had their

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common carotid arteries exposed bilaterally. The bilateral carotid arteries were clamped with aneurysmal clips. After a 10-min period of occlusion, the clips were removed allowing reperfusion of the blood. Sham operation was done in the same manner as the occlusion except for clamping the arteries. The body temperature was maintained at 37℃ with a heating pad during these treatments. Nebracetam or vehicle was given 10 min after reperfusion. In the treatment with nebracetam at 100 mg/kg, an additional group was given the drug at 60 min after reperfusion. The SHRSP with occlusion were anesthetized with diethylether and killed by decapitation 7 days after operation. The brain was immediately removed and frozen with isopentane at −30℃. Coronal brain slices, each with a thickness of 10 μm, were prepared and thaw-mounted sections were placed onto gelatin-coated glass slides, followed by staining with hematoxylin. The number of pyramidal cells per 1 mm linear length in an area selected at random for each section of the pyramidal cell layer of the hippocampal CA1 subfield (13) was calculated by counting the neurons showing neither necrotic nor shrunken morphology under a microscope with a scale attached to the eye lens. The average number of pyramidal cells obtained from two separate sections of each rat was taken up as the data for each rat. The effect of nebracetam on the blood pressure of SHRSP was examined by measuring systolic blood pressure according to the tail-cuff method using a pneumatic pulse transducer with an electrosphygmomanometer (Narco Biosystem Co., Houston, TX, USA), 1 day before, 1 hr after and 1 day after drug administration. Data are expressed as the means±S.E. Significance of the difference was determined by one-way analysis of variance followed by Dunnett's test for multiple comparisons.

In the hippocampal CA1 subfield, the number of pyramidal cells were 180.3±5.4 at 7 days after the sham-operation (Figs. 1A and 2). A 10-min bilateral carotid occlusion of SHRSP significantly decreased the number of pyramidal cells to 71.8±11.8 ($P<0.01$) at 7 days after the operation, demonstrating that 60% of the pyramidal cells was lost and/or degenerated by two-vessel occlusion of the SHRSP (Figs. 1B and 2). This event was delayed neuronal cell death, since neuronal degeneration in the pyramidal cell layer had not occurred at 12 hr, 1 day and 2 days after and then significantly occurred at 4 days after, leading to a peak at 7 days after ischemia (4). When SHRSP with occlusion were treated with 20 mg/kg of nebracetam 10 min after reperfusion, the injured pyramidal cells were not improved. The number of pyramidal cells in the nebracetam (20 mg/kg)-treated group was 25% less than that in the vehicle-treated group, although the change did not reach statistical significance. (Fig. 2). Nebracetam, given at the dose of 50 and 100 mg/kg at 10 min after

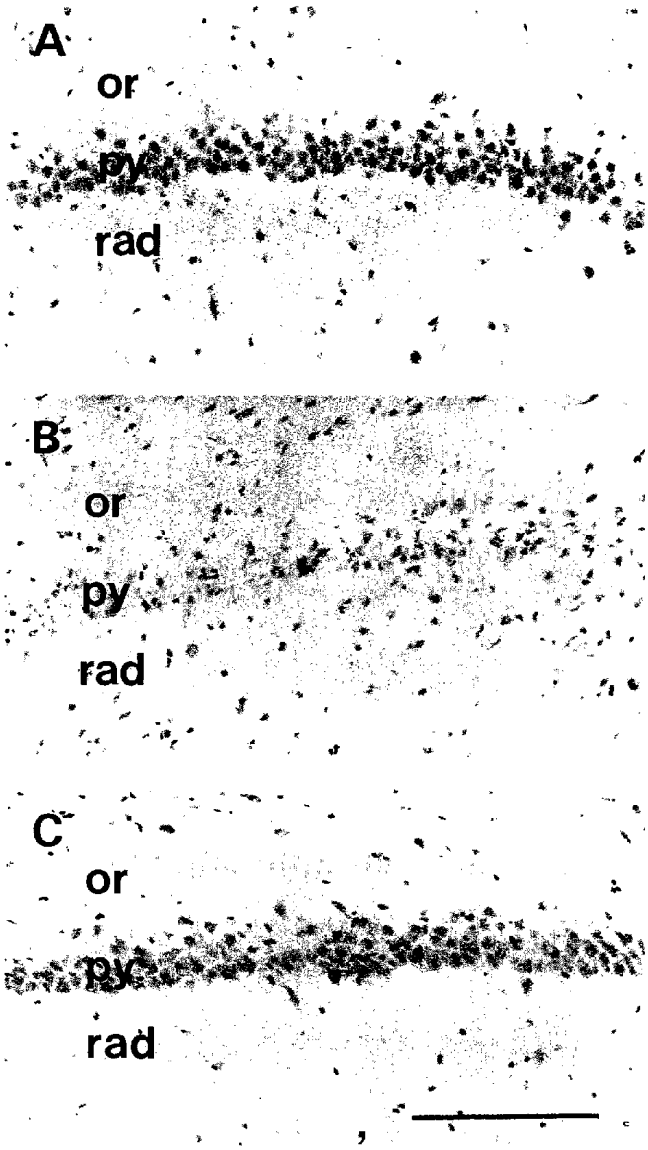

Fig. 1. Representative photographs showing the effect of nebracetam on ischemic neuronal damage in the hippocampal CA1 pyramidal cell layer of the SHRSP. Photographs of the hippocampal CA1 subfield were taken 7 days after the sham operation (A) and 10-min occlusion of bilateral common carotid arteries (B and C). Vehicle (B) and nebracetam at a dose of 100 mg/kg (C) were administered orally 10 min after reperfusion. or, stratum oriens; py, pyramidal cell layer; rad, stratum radiatum. Bar, 100 μm.

reperfusion, induced dose-dependent recovery of the occlusion-injured cells to the level of 30 and 50%, respectively, at 7 post-operative days (Figs. 1C and 2). When nebracetam at 100 mg/kg was administered to the SHRSP with occlusion 60 min after reperfusion, a moderate protective action was observed (23% recovery), an effect in contrast with that of nebracetam given 10 min after reperfusion (recovery of 50%) (Fig. 2). This finding means that drug administration at 10 min rather than 60

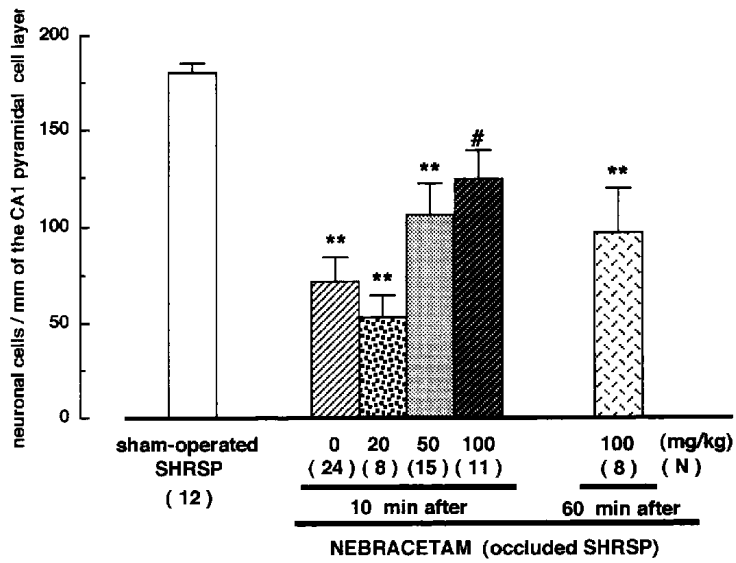

Fig. 2. Effect of nebracetam on the number of pyramidal cells per 1 mm linear length in the hippocampal CA1 subfield of the SHRSP with occlusion at 7 post-operative days. Nebracetam (20 and 50 mg/kg) was administered orally 10 min after reperfusion. Nebracetam at 100 mg/kg was given 10 or 60 min after reperfusion. $^{**}P<0.01$ vs the sham-operated SHRSP (Dunnett's test). $^{\#}P<0.05$, significant difference between nebracetam- and vehicle (dose=0)-treated group in the occluded SHRSP (Dunnett's test).

min after reperfusion is probably effective for the expression of the neuroprotective action of nebracetam in an in vivo study with rats. Nebracetam at the dose of 100 mg/kg, which is significantly effective in decreasing the ischemia-induced neuronal damage, did not alter the blood pressure of SHRSP with occlusion (1 day before, 229±6 mmHg; 1 hr after, 212±8 mmHg; 1 day after nebracetam, 227±4 mmHg). These findings resembled those in the SHRSP with occlusion treated with other doses of nebracetam (data not shown).

The present findings demonstrated that nebracetam dose-dependently prevents ischemic neuronal damage in the pyramidal cell layer of the hippocampal CA1 subfield in the two-vessel occlusion model of SHRSP. These findings indicate the usefulness of our occlusion model as a pharmacological tool to detect the neuroprotective action of the drug on ischemic brain injury. The neuroprotective action of nebracetam did not affect the blood pressure, suggesting that nebracetam protects against ischemic neuronal injury by interacting with neural tissue rather than the vascular system. The present findings are consistent with the previous in vitro findings indicating that nebracetam blocks striatal and hippocampal dysfunction induced by hypoglycemia/hypoxia (8, 10). Excessive Ca^{2+} entry, an etiological event of neuronal injury, occurs largely through Ca^{2+} channels gated by the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of the glutamate receptor, but also through voltage-gated Ca^{2+} channels (VGCC) (14). Nebracetam induced significant recovery from striatal dopaminergic impairment induced by L-glutamate and NMDA, but did not protect against that induced by BAY K 8644, an L-type VGCC agonist (9). These findings suggested that nebracetam possessed the ability to protect against striatal dysfunction by inhibiting an inappropriate Ca^{2+} influx through NMDA receptor-gated Ca^{2+} channels. This notion was supported by the evidence that nebracetam prevents the L-glutamate-evoked impairment of 2-deoxyglucose uptake in cortical slices (10). As demonstrated by our early work (8), nebracetam protected the striatal tissues from the functional damage produced by NaCN (cytochrome oxidase inhibitor) and olygomycin (mitochondrial ATP synthetase inhibitor). Therefore, it is likely that nebracetam stimulates oxidative phosphorylation by interacting with electron transport and the mitochondrial ATP synthesizing complex, leading to an attenuation of the marked breakdown of ATP evoked by ischemia (8). These lines of evidence together indicate that an activation of energy metabolism and an inhibition of NMDA receptor-mediated Ca^{2+} delivery is likely to be operative in the mechanisms mediating the neuroprotective action of nebracetam on ischemic neuronal cell death in the hippocampus.

In conclusion, the present findings with the two-vessel occlusion model of SHRSP provide evidence that nebracetam has a neuroprotective action on the delayed neuronal cell death evoked by transient forebrain ischemia in the pyramidal cell layer of the hippocampal CA1 subfield. This evidence supports our previous notion that

nebracetam may be useful for the treatment of hypoxic/ischemic brain injury (8, 9).

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

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