Neuroprotective Effects of Citidine-5-diphosphocholine on Impaired Spatial Memory in a Rat Model of Cerebrovascular Dementia

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Received January 20, 2011; Accepted April 25, 2011

Abstract. Citidine-5-diphosphocholine or citicoline (CDP-choline) is used as a neuroprotective and memory-enhancing drug in cerebral stroke, Alzheimer’s disease, and other neurovascular diseases. Non-clinical studies have demonstrated the neuroprotective effects of CDP-choline in ischemic animal models. However, the relationship between the neuroprotective effect and the memory enhancing effect of CDP-choline is still unknown. No studies have demonstrated the ameliorative effect on impaired spatial memory and the suppressive effect on neuronal cell death of CDP-choline in the same model. In this study, we examined the effect of CDP-choline on impaired spatial memory and hippocampal CA1 neuronal death in rats subjected to repeated cerebral ischemia, and we compared the mechanism of CDP-choline to that of donepezil. Seven days post administration of CDP-choline (100, 300, 1000 mg/kg per day, p.o.) or donepezil increased correct choices and reduced error choices in an eight-arm radial maze task in a dose-dependent manner. Neuronal cell death of caspase-3 protein–positive neurons in the hippocampus were reduced by repeated administration of CDP-choline at the highest dose. These results suggest that CDP-choline has ameliorative effects on the impairment of spatial memory via hippocampal neuronal cell death in a rat model of cerebral ischemia.

Keywords: citidine-5-diphosphocholine (CDP-choline), spatial memory, repeated ischemia, apoptosis

Introduction

Citidine-5-diphosphocholine or citicoline (CDP-choline) is used as a neuroprotective and memory enhancing drug in conditions such as cerebral stroke (1, 2), Alzheimer’s disease (3 – 5), and other neurovascular diseases (6). A meta-analysis of double-blind, placebo-controlled, randomized trials of CDP-choline for cognitive impairment due to cerebrovascular dementia revealed a positive effect on memory and behavior (7). It is thought that CDP-choline is used as a raw material for the synthesis of phospholipids, which are cell-membrane components required to maintain the structure of nerve cells. When ischemia consumes ATP and drives an increase in the amount of AMP, there is a release of free fatty acids, such as arachidonic acid and diacylglycerols from phosphatidylcholine, and further lesions are formed. Citicoline traps the cytotoxic free fatty acids and diacylglycerols by stimulating phosphatidylcholine synthesis, suppressing the occurrence of lesions (8).

Non-clinical studies have also demonstrated the neuroprotective effects of CDP-choline in ischemic animal models. In a gerbil forebrain ischemia model, application of CDP-choline increased the number of surviving cells in the hippocampal CA1 region (9). Intraperitoneal injection of CDP-choline significantly decreased the number of cells that were immunohistopathologically positive for active caspase-3 and DNA fragmentation in the penum-
Effect of CDP-Choline on Spatial Memory

In addition, a memory enhancing effect of CDP-choline has been reported in aged rats and mice (11–14). However, the relationship between the neuroprotective effect and the memory enhancing effect of CDP-choline is still unknown.

We previously reported that rats subjected to repeated cerebral ischemia could be used as a model of cerebrovascular dementia (15, 16). We have found a correlation between spatial memory impairment and hippocampal CA1 neuronal death, both of which were induced by two 10-min periods of cerebral ischemia with an interval of 1 h (15, 16). This model could be useful in the development of new drugs for the treatment of cerebrovascular dementia.

Donepezil is known to ameliorate spatial memory impairment as an acetylcholine esterase inhibitor (17), and some investigators reported donepezil also has neuroprotective effects (18–20). However, the quantitative evaluation of donepezil on neuronal cell death in repeatedly ischemic rats is still unclear. In this study, we examined the effect of CDP-choline on spatial memory impairment and hippocampal CA1 neuronal death in rats subjected to repeated cerebral ischemia, and we compared the mechanism of CDP-choline to that of donepezil.

Materials and Methods

Materials

CDP-choline (Cognizin®) was supplied by KYOWA HAKKO BIO CO., LTD. (Tokyo), and donepezil was supplied by Eisai Co., Ltd. (Tokyo).

Animals

Male Wistar rats weighing 250–300 g were purchased from Kyudo Co., Ltd. (Saga). They were housed in groups of 4 to 5 per cage in a temperature-controlled room (23 ± 2°C) with a relative humidity of 60 ± 10% and were maintained under a 12-h light–dark cycle. All procedures regarding animal care and use were carried out based on the regulations dictated by the Experimental Animal Care and Use Committee of Fukuoka University.

Eight-arm radial maze task

Behavioral testing using an eight-arm radial maze task (Neuroscience Co., Tokyo) was conducted as previously reported (21). The maze consisted of a central platform (24 cm in diameter) with eight arms that extended radially. Rats were allowed to visit each arm to eat eight pellets in food cups located near the end of each arm. Each test animal was trained once per day to memorize the apparatus. The performance of test animals in each trial was assessed using two parameters: number of correct choices in the initial eight chosen arms, and number of errors (defined as choosing arms that had already been visited). When the test animals made seven or eight correct choices and no more than one error in three successive sessions, they were deemed to have memorized the maze. In other words, the rats had acquired spatial memory of the eight-arm radial maze.

Repeated cerebral ischemia and drug administration

The rats that had been trained in the eight-arm radial maze task and acquired spatial memory were subjected to repeated ischemia induced according to our previous protocol (21). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Tokyo Kasei, Tokyo), and then both vertebral arteries were electrocauterized and the common carotid arteries were exposed. On the next day, repeated ischemia was induced for 10 min by occluding both common carotid arteries using aneurysm clips, and this was repeated once after 1 h. These rats are referred to as repeated ischemic rats. The body temperature was maintained at 37°C using a heat pad and a heat lamp during the operation and occlusion until the righting reflex reappeared. The rats that failed to demonstrate loss of the righting reflex during occlusion were excluded from subsequent experiments. We used rats with both vertebral arteries electrocauterized and both common carotid arteries exposed, but without being occluded, as sham-operated controls. The oral administration of CDP-choline or donepezil was performed 1 h after the first occlusion, and the administration was continued for 6 d. The last administration was performed 1 h before the behavioral experiments. Distilled water was administered orally to the sham-operated group at the same time points.

Histological examination

Rats were sacrificed after the behavioral test by deep anesthetization with pentobarbital (50 mg/kg, i.p.) and transcardial perfusion with cold heparinized saline, followed by perfusion of 4% paraformaldehyde. Brains were removed and postfixed overnight in paraformaldehyde, before being dehydrated and embedded in paraffin. Representative coronal sections (5 μm), including the dorsal hippocampus, were obtained using a rotary microtome and stained with hematoxylin and eosin (HE). Microscopic examination of the neuronal density of the CA1 region was carried out to detect neuronal damage in the hippocampus.

Caspase-3 measurement

Rats were sacrificed after the behavioral test by deep anesthetization with pentobarbital (50 mg/kg, i.p.). Hip-
pocampi were separated by dissection and were homogenized in lysis buffer (10 mL Tris buffer saline, 100 μL 100 mM EDTA, 100 μL 100 mM EGTA, 100 μL Triton X-100, and 100 μL lyophilized protease inhibitor cocktail; Nacalai Tesque, Kyoto), and centrifuged (4°C, 20,400 × g, 30 min) to extract the protein. Protein concentrations were determined by the Bradford method. Equal amounts of protein were separated by 15% SDS-PAGE (Bio-Rad, Hercules, CA, USA), and then they were transferred to 0.22-μm PVDF membranes using a Trans-Blot semi-dry system (Bio-Rad). The membranes were blocked in 5% skim milk in Tris-buffered saline with Tween buffer for 1 h and then incubated overnight at 4°C with the following primary antibodies: anti-caspase-3 (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin (1:5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, the membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibody (bovine anti-rabbit IgG, 1:10000; Cell Signaling Technology, Inc.) for 2 h at room temperature. The blots were developed using a chemiluminescence kit (GE Healthcare, Hertfordshire, UK) and were exposed to film. The bands on the film were scanned and analyzed with Image J.

**Statistical analysis**

Data are expressed as the mean ± S.E.M. Statistical significance within each group was estimated using the F-test followed by the Student’s t-test. When the experimental series involved more than two groups, one-way analysis of variance was used, with a post-hoc Dunnett’s test. P-values of less than 0.05 were considered statistically significant.

**Results**

**Influence of 7-day post-ischemic treatment with CDP-choline on spatial memory in an eight-arm radial maze**

Rats subjected to repeated ischemia showed a decrease in the number of correct choices and an increase in the number of errors compared with sham-operated rats (Fig. 1). Post-ischemic administrations of CDP-choline for 7 days significantly increased the number of correct choices and decreased the number of errors (Fig. 1). Similarly, 7-day post-ischemic administration of donepezil significantly increased the number of correct choices and decreased the number of errors in the eight-arm radial maze (Fig. 1).

**HE staining and caspase-3 expression in the hippocampus**

HE staining revealed pyknosis, eosinophilia, karyorrhexis, and chromosome condensation in the CA1 pyramidal neurons of the vehicle-treated repeated ischemia.
group compared to the sham group (Fig. 2: A, B). Repeated cerebral ischemia induced cell death among hippocampal CA1 pyramidal neurons. Donepezil did not affect the ischemia-induced neuronal cell death (Fig. 2C) and the amount of caspase-3 in the hippocampus (Fig. 3). In contrast, post-ischemic treatment with CDP-choline significantly suppressed neuronal death (Fig. 2: D, E and F). In repeated ischemia rats, caspase-3 was significantly increased compared to sham-operated rats (Fig. 3). Post-ischemic administrations of CDP-choline for 7 days significantly decreased the amount of caspase-3 in the hippocampus (Fig. 3).

**Discussion**

In the present study, CDP-choline improved the impaired spatial memory in ischemic rats subjected to an eight-armed radial maze task and demonstrated neuroprotective effects in the hippocampal CA1 region. To the best of our knowledge, this is the first study demonstrating CDP-choline ameliorative effects on impaired spatial memory.
memory and suppressive effects on neuronal cell death in the same model. These results suggest beneficial effects of CDP-choline on impaired memory in cerebrovascular dementia follows the neuroprotective effects.

As reported in previous studies using a rat model of cerebral ischemia, spatial memory is impaired 6 days after repeated ischemia in the eight-arm radial maze task. At the same time, neuronal cell death occurs in the hippocampus (15, 16, 21, 22). In the present study, post-ischemic administration of CDP-choline (100, 300, 1000 mg/kg) for 7 days significantly increased the number of correct choices and decreased the number of errors in a dose-dependent manner. Moreover, repeated administration of CDP-choline reduced caspase-3 in the hippocampus. These results suggest that chronic treatment with CDP-choline suppresses neuronal cell death and improves spatial memory. CDP-choline, in addition to its well-known action of stabilizing the cell membrane (23, 24) and reducing lipid peroxidation in an ischemia reperfusion model (25), has been shown to reduce the expression of cleaved caspase-3 in the penumbra area cells, as well as reduce the number of cells bearing nuclear DNA fragmentation after middle cerebral artery occlusion (26). Thus, it has been suggested that CDP-choline could restore impaired spatial memory by its suppressive effects on neuronal cell death. It has not been reported that CDP-choline has acetylcholine-releasing effects (including effects on activity or amounts of acetylcholine esterase), as has been reported for donepezil. However, it is not ruled out that CDP-choline could act as a choline donor in the central nervous system, due to CDP-choline–enhanced acetylcholine availability (27–29).

In this study, the efficacy of CDP-choline and donepezil on the number of errors in eight-arm radial maze were not perfect. In comparison with the report of researchers indicating the effects of CDP-choline on ischemic injury, the dose used in this report is not a low dose (9, 10, 23), and a previous study reported that 3 mg/kg of donepezil significantly improved the spatial memory impairment induced by the combination of β-amyloid and cerebral ischemia (30). The improvement effect of 1000 mg/kg of CDP-choline on the impaired memory by ischemia is not inferior to that of 3 mg/kg of donepezil, which was therefore thought to be sufficient to exert the ameliorative effect on memory impairment.

In this study, donepezil improved spatial memory in rats subjected to repeated cerebral ischemia. Donepezil powerfully inhibits acetylcholinesterase (17) and elevates acetylcholine concentration in the synapse, which results in increased cholinergic transmission. Previous studies have suggested the donepezil has neuroprotective effects aside from its acetylcholinesterase-inhibiting action through up-regulation of Bel-2, an anti-apoptotic protein (18), stimulation of nicotinic acetylcholine receptors (19), and inhibition of glycogen synthase kinase-3 (GSK-3) activity (20). It is unknown whether donepezil exerts its neuroprotective effect in rats subjected to repeated cerebral ischemia. Induction of the neuroprotective effects of donepezil via the nicotinic acetylcholine receptor in rats subjected to repeated cerebral ischemia may require a higher concentration and/or long-term treatment because the concentration of donepezil showing neuroprotection is higher than the concentration needed to exhibit acetylcholinesterase-inhibiting action in vitro (17, 19, 20).

In conclusion, our results demonstrate that CDP-choline has ameliorative effects on impaired spatial memory in a rat model of cerebral ischemia via hippocampal neuronal death.

Acknowledgment

This study was partially supported by a research grant from KYOWA HAKKO BIO CO., LTD.

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