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Protective effect of donepezil in a primary culture of rat cortical neurons exposed to oxygen–glucose deprivation

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Abstract

Donepezil hydrochloride (donepezil: (±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride) is a potent acetylcholinesterase inhibitor used for treatment of Alzheimer's disease. Although acetylcholinesterase inhibitors are used as a symptomatic treatment for Alzheimer's disease, it is not clear whether or not they are effective against progressive degeneration of neuronal cells. In this study, we investigated the neuroprotective effects of donepezil and other acetylcholinesterase inhibitors used for treatment of Alzheimer's disease, i.e., galantamine, rivastigmine, and tacrine. As a neurodegenerative model, we used rat cortical neurons exposed to oxygen–glucose deprivation. Lactate dehydrogenase (LDH) released into the culture medium was measured as a marker of neuronal cell damage. First, the effects of donepezil (10 μM) on three different treatment schedules (from 12 h before to 24 h after oxygen–glucose deprivation (pre-12 h), from 1 h before to 24 h after oxygen–glucose deprivation (pre-1 h) and from 1 h after to 24 h after oxygen–glucose deprivation (post-1 h)) were compared. The pre-12-h treatment most effectively inhibited LDH release. The protective effect of donepezil was confirmed morphologically. Next, the effects of donepezil and the other three acetylcholinesterase inhibitors were compared under the pre-12-h treatment condition. Donepezil (0.1, 1, and 10 μM) significantly decreased LDH release in a concentration-dependent manner. However, galantamine (1, 10, and 100 μM), tacrine (0.1, 1, and 10 μM), and rivastigmine (0.1, 1, and 10 μM) did not significantly decrease LDH release. The neuroprotective effect of donepezil was not antagonized by scopolamine or mecamylamine. These results demonstrate that donepezil has a protective effect against oxygen–glucose deprivation-induced injury to rat primary cultured cerebral cortical neurons. Besides, it is suggested that this effect of donepezil is independent of muscarinic cholinergic system and nicotinic cholinergic system. Thus, donepezil is expected to have a protective effect against progressive degeneration of brain neuronal cells in ischemic cerebrovascular disease and Alzheimer's disease.

Keywords: Donepezil; Acetylcholinesterase inhibitor; Oxygen–glucose deprivation; LDH release; Primary cultured cerebral cortical neuron; (Rat)

1. Introduction

Senile dementia is becoming an increasingly important medical and social problem in many developed countries. Dementia in elderly subjects falls into two main categories, i.e., Alzheimer's disease and cerebrovascular disease. Pathologically, Alzheimer's disease is characterized by excessive accumulation of senile plaques and neurofibrillary tangles in the brain. In contrast, vascular dementia, which is dementia associated with cerebrovascular disease, is caused by arteriosclerosis, white matter ischemic change, or large vessel stroke resulting in multiple lacunar infarcts (de la Torre, 2002).

Acetylcholinesterase inhibitors have been developed as promising agents for the palliative therapy of Alzheimer's disease (Peskind, 1998; Giacobini, 2000), because there is increasing neurochemical and pharmacological evidence that cognitive decline in Alzheimer's disease is linked to cholinergic deficiency (Benzi and Moretti, 1998). So far, four acetylcholinesterase inhibitors have been approved by the FDA in the USA. 9-Amino-1,2,3,4-tetrahydroacridine (tacrine) was the first to be approved (in 1993) for the treatment of Alzheimer's disease, although its use is limited due to its hepatic toxicity and undesirable effects on the peripheral nervous system. Therefore, novel acetylcholinesterase inhibitors, which are more tolerable and effective, have been developed. (±)-2-[(1-Benzylpiperidin-4-yl)methyl]-5,6-

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dimethoxyindan-1-one monohydrochloride (donepezil) is a novel, piperidine-based, acetylcholinesterase inhibitor (Sugimoto et al., 1995), which was approved in 1996. (*S*)-*N*-Ethyl-3-[(1-dimethylamino)ethyl]-*N*-methylphenylcarbamate hydrogentartrate (rivastigmine) is a carbamate-based acetylcholinesterase inhibitor that was approved in 2000. 4a,5,9,10,11,12-Hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-ol (galantamine) is a naturally occurring amaryllidaceae alkaloid, and this drug was approved in 2001.

Considering the symptom-ameliorating effect of acetylcholinesterase inhibitors on cognitive deficits of Alzheimer's disease, it seems reasonable to suppose that acetylcholinesterase inhibitors would probably also be effective on the cognitive decline in vascular dementia. Some clinical studies have suggested the usefulness of acetylcholinesterase inhibitors for the treatment of vascular dementia (Mendez et al., 1999; Pratt et al., 2002). Although it is known that acetylcholinesterase inhibitors ameliorate cognitive dysfunction in Alzheimer's disease (and vascular dementia) by temporarily activating undamaged cholinergic neurons, it remains to be established whether these drugs also interrupt the progress of mental decline by inhibiting subsequent neuronal cell death.

Substantial evidence now indicates that sporadic Alzheimer's disease is initiated by vascular factors that precede the neurodegenerative process (de la Torre, 2002). For instance, epidemiological studies show that practically all risk factors for Alzheimer's disease reported thus far have a vascular component that reduces cerebral perfusion, and the risk factors for Alzheimer's disease are associated with those for cerebrovascular disease (Breteler, 2000a,b; de la Torre, 1997). Moreover, individuals found at postmortem examination to have definitive neuropathologic evidence of Alzheimer's disease and brain infarcts are more likely to have had severe cognitive impairment and dementia than those found to have evidence of Alzheimer's disease without brain infarcts (Hofman et al., 1997). These findings are consistent with the idea that drugs, which inhibit neuronal degeneration induced by ischemic events, may also inhibit the progressive mental decline in Alzheimer's disease and vascular dementia.

Acetylcholine is known to potentiate the effect of nerve growth factor (NGF) (Knipper et al., 1994), and the neuroprotective effect of NGF against free radical insult is well documented (Jackson et al., 1990). Thus, acetylcholinesterase inhibitors are expected to have neuroprotective effects against ischemia-induced injury. There is evidence that some acetylcholinesterase inhibitors prevent ischemia-induced cell injury. Han et al. (2000) reported that bis(7)-tacrine could protect astrocytes against ischemia-induced cell injury, and Zhou et al. (2001) showed that huperzine A and donepezil could protect rat pheochromocytoma (PC12) cells against ischemia-induced cell injury. However, these data were obtained not with neuronal cells, but with astrocytes or cell lines, and there is no report so far to show that acetylcholinesterase inhibitors prevent neuronal cell degeneration induced by ischemia. Primary cultured neurons injured by oxygen–glucose deprivation provide a useful in vitro ischemia model to evaluate the anti-ischemic effect of drugs (Kimura et al., 1998).

In this study, therefore, we investigated the neuroprotective effects of donepezil and other acetylcholinesterase inhibitors, galantamine, rivastigmine, and tacrine, against oxygen–glucose deprivation-induced damage, using rat cerebral cortex primary cultured neurons.

2. Materials and methods

2.1. Drugs

Donepezil and rivastigmine were supplied by Eisai (Ibaraki, Japan). Tacrine, (−)-scopolamine methylbromide (scopolamine), and mecamylamine hydrochloride (mecamylamine) were purchased from Sigma (St. Louis, MO, USA). Galantamine and dizocilpine (MK801) were obtained from Tocris (Ballwin, MO, USA), and Research Biochemicals (Natick, MA, USA), respectively. Before use, these drugs were dissolved in distilled water and then further diluted with Krebs–Ringer bicarbonate buffer (KR buffer), or Dulbecco's modified essential medium (DMEM) to yield the desired final concentrations.

2.2. Cortical cell cultures

Cortical cell cultures were prepared from fetal rats of the Wistar strain (gestational age of 17–18 days, Charles River Japan, Kanagawa, Japan) according to the procedures described previously (Ohgoh et al., 1998). Briefly, the cortex was dissected and kept in ice-cold Hank's balanced salt solution (HBSS; 10 mM HEPES, pH 7.3) (Gibco, NY, USA) and then incubated at 37 °C for 30 min in Ca^{2+}/Mg^{2+}-free HBSS containing 0.25% trypsin (Gibco) and 0.2 mg/ml deoxyribonuclease (Sigma). The cortical tissues were dissociated to single cells by gentle trituration. The cell suspension was mixed with DMEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 10% heat-inactivated horse serum (Gibco), 5 μg/ml insulin (Sigma), 30 nM sodium selenite (Sigma), 100 μM putrecine (Sigma), 20 nM progesterone (Sigma), 15 nM biotin (Sigma), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 8 mM glucose, and 1 mM pyruvic acid (Sigma), as described before (Scholtz et al., 1988). The cell suspension was centrifuged at 310 × *g* for 3 min, and the resulting pellets were resuspended in the medium described above and plated onto cover slips coated with poly-L-lysine (Sigma). The cells were cultured in a CO_2 incubator (5% [v/v], 37 °C) for 1 day, and the cover slips were then transferred onto confluent glial cell layers and cultured for 2 days in DMEM containing 5% fetal calf serum and the same supplements as described above. Then, the cortical

cells were treated with 10 μM cytosine arabinofuranoside (Sigma) for 12 h (it was added to the culture medium 3 days after plating) to reduce the growth of contaminating non-neuronal cells. Seven days after plating, the cortical cells were cultured in DMEM containing 5% fetal calf serum and the same supplements as described above, but without glutamine. The culture medium, which was conditioned with glial cells for 1 day, was changed every 3–4 days.

The glial cells used were obtained from the cerebral cortex of fetal rats of the Wistar strain (gestational age of 17–18 days). The cerebral cortex was dissected and triturated in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The glial cells were cultured in the CO_2 incubator for 5–7 days before use. All experiments were approved by the Animal Care and Use Committee of Eisai.

2.3. Oxygen–glucose deprivation

As shown previously (Kimura et al., 1998), cultured neurons were damaged by exposure to oxygen–glucose deprivation. The oxygen–glucose deprivation treatment was performed on 10-day-old cultures. Cortical neurons cultured on cover slips were transferred to new plates containing glucose-free KR buffer with the following ionic composition: 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 137 mM NaCl, 4.17 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, and 10 mM HEPES (pH 7.4). The plates were transferred to a CO_2 incubator for 1 h. Then, the cultures were placed in an anaerobic chamber containing 95% (v/v) N_2 and 5% (v/v) CO_2 and exposed to oxygen–glucose deprivation for 40–50 min at 37 °C. Exposure time to oxygen–glucose deprivation was adjusted in order to control LDH release to around 15% of total LDH. The oxygen–glucose deprivation was terminated by replacing the KR buffer with DMEM containing the supplements described above conditioned with glial cells for 1 day, and the cultures were returned to the CO_2 incubator for 24 h.

2.4. LDH assay

LDH efflux into the culture medium was measured immediately after the oxygen–glucose deprivation period and 24 h after the oxygen–glucose deprivation period in all experiments. Remaining LDH in the cells was lysed with 0.1 M phosphate buffer (0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4; pH 7.4) containing 0.5% Triton X-100 and was measured 25 h after the oxygen–glucose deprivation period. LDH release as an indicator of neuronal injury was represented by the percentage leakage of LDH into the culture medium, with respect to the total LDH activity per culture (total LDH efflux/total LDH measured). The decrease of NADH was spectrophotometrically monitored at 340 nm as LDH activity (Koh and Choi, 1987).

2.5. Drug administration schedules

Donepezil at the concentration of 10 μM was added to the cells according to the following three schedules to identify the optimal treatment schedule. First, the cells were treated with donepezil from 12 h before to 24 h after oxygen–glucose deprivation (pre-12 h). Second, the cells were treated with donepezil from 1 h before to 24 h after oxygen–glucose deprivation (pre-1 h). Third, the cells were treated with donepezil from 1 h after to 24 h after oxygen–glucose deprivation (post-1 h). Other acetylcholinesterase inhibitors were applied to the cultures on the pre-12-h schedule. Scopolamine and mecamylamine were applied to the cultures with the same timing as donepezil.

2.6. Statistical analysis

The data were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test using the software package SAS ver. 6.12 (SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Influence of administration schedule on the neuroprotective effect of donepezil against ischemic injury

As shown in Fig. 1, donepezil on the pre-12-h schedule dramatically decreased the LDH release. The pre-1-h schedule also significantly decreased the LDH release, but less effectively than the pre-12-h schedule. On the other hand, donepezil on the post-1-h schedule did not significantly

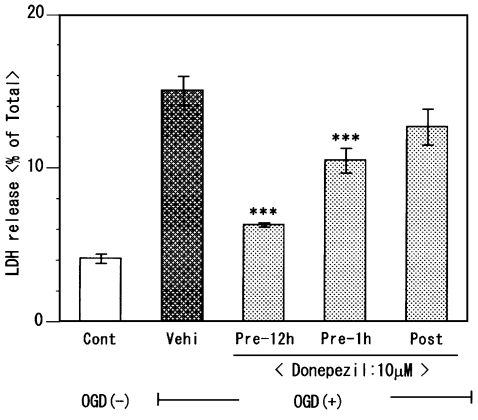

Fig. 1. Comparison of the effects of donepezil on three different treatment schedules, pre-12 h, pre-1 h and post-1 h, on LDH release induced by oxygen–glucose deprivation in rat cultured cerebral cortical neurons. OGD: oxygen–glucose deprivation. OGD(−): oxygen–glucose deprivation nontreated cells. OGD(+): oxygen–glucose deprivation-treated cells. Cont: oxygen–glucose deprivation nontreated control. Vehi: vehicle. Pre-12 h: treatment from 12 h before to 24 h after oxygen–glucose deprivation. Pre-1 h: treatment from 1 h before to 24 h after oxygen–glucose deprivation. Post: treatment from 1 h after to 24 h after oxygen–glucose deprivation. Values are means ± S.E. $N=6$. ***$P<0.005$ compared with vehicle group (Dunnett's multiple comparison).

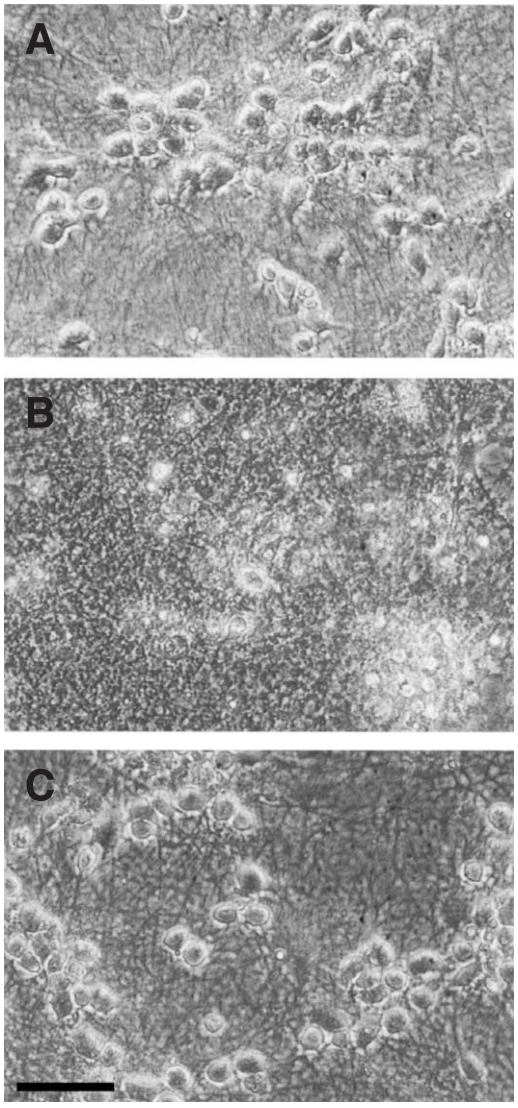

Fig. 2. Effect of donepezil on the morphology of rat cultured cerebral cortical neurons. (A) Oxygen–glucose deprivation nontreated cells. (B) Cells exposed to oxygen–glucose deprivation. (C) Cells treated with donepezil (10 μM) from 12 h before to 24 h after oxygen–glucose deprivation. Scale bar = 0.1 mm.

decrease the LDH release. Therefore, subsequent experiments were done with the pre-12-h schedule for all the drugs tested.

3.2. Effect of donepezil on the morphology of cultured neurons exposed to oxygen–glucose deprivation

Fig. 2 shows typical photographs of normal rat cultured cerebral cortical neurons (A) and those exposed to oxygen–glucose deprivation (B and C). Visual inspection using a phase-contrast microscopy indicated that the normal cells were viable (Fig. 2A). Many cells (though not all) exposed to oxygen–glucose deprivation showed obvious cell body damage, shrinkage, and cellular debris (Fig. 2B). In contrast, the cells treated with donepezil (10 μM) on the pre-12-h schedule were much better preserved and did not show damage like that of Fig. 2B. Thus, donepezil appears to have a protective effect against oxygen–glucose deprivation-induced injury (Fig. 2C).

3.3. Comparison of neuroprotective effects of donepezil with other acetylcholinesterase inhibitors

The effects of the four acetylcholinesterase inhibitors were evaluated in two experiments, one with donepezil and galantamine (Fig. 3A) and the other with tacrine and rivastigmine (Fig. 3B). In order to reduce the inter-experimental variance, LDH release of each vehicle-treated group were controlled to the same level (A: 16.6%; B: 17.1%). The effects of the positive control, MK801 (1 μM, evaluated with pre-1-h treatment), were almost the same in the two experiments.

As shown in Fig. 3A, donepezil (0.1–10 μM) significantly decreased the LDH release in a concentration-depen-

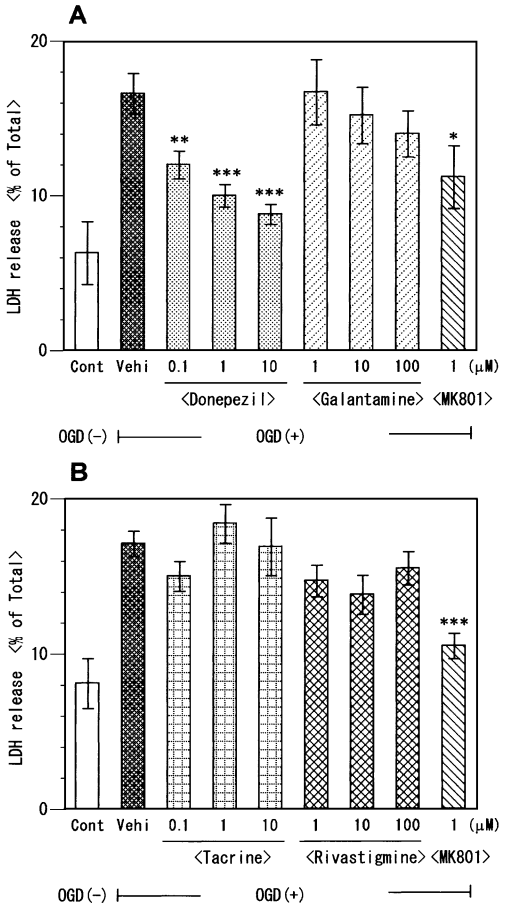

Fig. 3. Effects of donepezil, galantamine, tacrine, and rivastigmine on LDH release induced by oxygen–glucose deprivation in rat cultured cortical neurons. All acetylcholinesterase inhibitors were given on the pre-12-h schedule (see text). MK-801, which was used as a positive control, was given on the pre-1-h schedule. OGD(−): oxygen–glucose deprivation nontreated cells. OGD(+): oxygen–glucose deprivation-treated cells. Cont: oxygen–glucose deprivation nontreated control. Vehi: vehicle. Values are means ± S.E. $N=6$. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ compared with vehicle group (Dunnett's multiple comparison).

dent manner. At 1 and 10 μM, donepezil was more potent than the positive control, MK-801. Galantamine (1–100 μM) tended to reduce the LDH release in a concentration-dependent manner, but the effect was not statistically significant. Fig. 3B shows the effects of tacrine (0.1–10 μM) and rivastigmine (0.1–10 μM). Neither of these drugs significantly decreased the LDH release. Thus, only donepezil among the four acetylcholinesterase inhibitors showed a clear neuroprotective effect against oxygen–glucose deprivation-induced ischemic injury.

3.4. Influence of acetylcholine receptor antagonists on the neuroprotective effect of donepezil

As shown in Fig. 4A and B, neither a muscarinic receptor antagonist, scopolamine (10 μM), nor a nicotinic receptor antagonist, mecamylamine (10 μM), influenced LDH release when applied alone. Moreover, these antagonists had no effect on the LDH release ratio in donepezil-treated cells. Therefore, these acetylcholine receptor antagonists had no influence on the neuroprotective effect of donepezil.

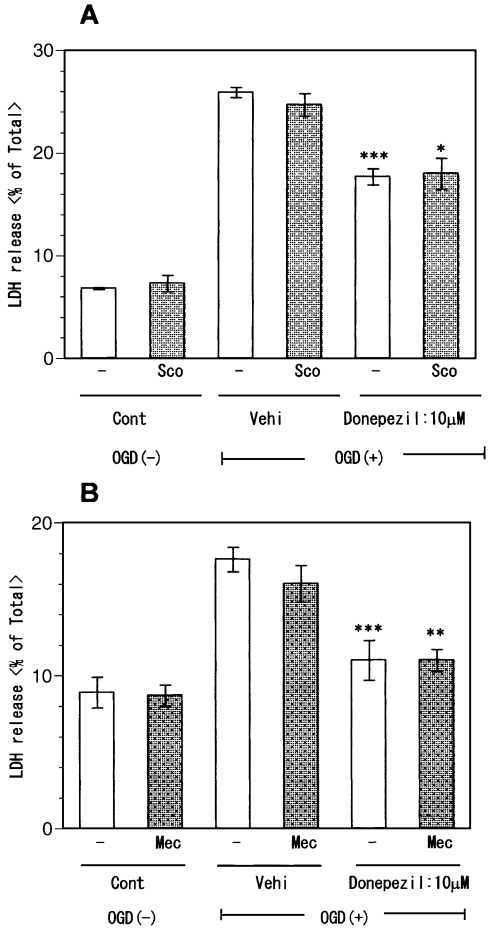

Fig. 4. Effects of donepezil, scopolamine, and mecamylamine on LDH release induced by oxygen–glucose deprivation in rat cultured cortical neurons. All compounds were given on the pre-12-h schedule (see text). OGD(−): oxygen–glucose deprivation nontreated cells. OGD(+): oxygen–glucose deprivation-treated cells. Cont: oxygen–glucose deprivation nontreated control. Vehi: vehicle. Sco: cells treated with scopolamine (10 μM). Mec: cells treated with mecamylamine (10 μM). Values are means ± S.E. $N=4$ to 6, respectively. *$P<0.05$, **$P<0.01$, and ***$P<0.005$ compared with vehicle group (Dunnett's multiple comparison).

4. Discussion

The present results demonstrate that donepezil has a neuroprotective effect against ischemia-induced neuronal cell injury. Although donepezil is known to alleviate oxygen–glucose deprivation-induced injury in the PC12 cells (Zhou et al., 2001), the present study is the first to demonstrate a protective effect in neurons.

Donepezil is a well-tolerated drug that improves cognitive and global functions in patients with mild to moderate Alzheimer's disease. Moreover, similar findings were obtained in patients with vascular dementia in a randomized, double-blind, placebo-controlled trial (Pratt et al., 2002). The mechanism of donepezil action is believed to involve temporary activation of the performance of undamaged cholinergic neurons in the brain by increasing acetylcholine concentration in the synaptic cleft. However, the present results imply that donepezil may also ameliorate progressive mental decline by inhibiting subsequent neuronal cell death.

A substantial body of evidence indicates that sporadic Alzheimer's disease is initiated by vascular factors that precede the neurodegenerative process (de la Torre, 2002). Indeed, epidemiological studies show that practically all risk factors for Alzheimer's disease reported thus far have a vascular component that reduces cerebral perfusion, and these risk factors for Alzheimer's disease are closely related to those for cerebrovascular disease (Breteler, 2000a,b; de la Torre, 1997). Approximately 30% of all Alzheimer's disease brains show some form of cerebrovascular pathology, and practically all Alzheimer's disease brains exhibit either periventricular white matter lesions, microvessel degeneration, cerebral amyloid angiopathy, or combinations of these lesions (Kalaria and Ballard, 1999; Olichney et al., 1996; Premkumar et al., 1996). Therefore, drugs that rescue neuronal cells from ischemic damage induced by cerebrovascular pathology should at least delay the further impairment of brain function in Alzheimer's disease.

In the case of vascular dementia, the relationship between disturbance of intellectual function and brain ischemia is more direct than in the case of Alzheimer's disease. Ischemia is induced by atherosclerosis and arterial occlusion, which lead to neuronal degeneration and infarction. The disturbance of brain function directly induced by the infarcts is likely to be a cause of cognitive impairment in vascular dementia. Moreover, recurrent brain ischemia may aggravate the symptoms of vascular dementia. Thus, administration of a drug with anti-ischemic activity might delay or prevent the deterioration of cognitive function in vascular dementia, as well as in Alzheimer's disease.

When trying to predict clinical efficacy by extrapolating in vitro activity, it is important to consider the concentration of the drug that is clinically achievable. Pharmacokinetic study suggests that the clinically achievable concentration of donepezil is similar to the concentration of the drug used in this study. Plasma concentration (C_{max}) of donepezil in the steady state in patients repeatedly treated with this drug at the oral dose of 10 mg/kg for 28 days was 60.5 ± 10.0 ng/ml (0.15 μM) (Tiseo et al., 1998). Moreover, an animal study indicated that the concentration of the drug in the brain was 5–10 times higher than that in plasma (Kosasa et al., 2000). Thus, the neuroprotective effect of donepezil (0.1–10 μM) against ischemic injury observed in this study could be manifested in the clinical situation.

It has been reported that cholinesterase inhibitors have protective effects against ischemia-induced cell injury (Han et al., 2000; Zhou et al., 2001), so the neuroprotective effect of donepezil might be due to acetylcholinesterase inhibition. Thus, we examined the effects of four acetylcholinesterase inhibitors, donepezil, galantamine, tacrine, and rivastigmine under the same experimental conditions. The effect of donepezil was dose dependent. Donepezil significantly decreased LDH release even at the lowest concentration of 0.1 μM. However, galantamine did not decrease LDH release even at the highest concentration of 100 μM (Fig. 3A). It is not unreasonable that donepezil showed a more than 1000 times greater neuroprotective effect than galantamine, because donepezil has approximately 200 times more potent inhibitory activity on rat brain acetylcholinesterase compared with galantamine (the IC_{50} values are 6.7 ± 0.35 nM (Ogura et al., 2000) and 1200 ± 33 nM [unpublished data], respectively). However, unexpectedly, neither tacrine (0.1–10 μM) nor rivastigmine (0.1–10 μM) significantly decreased LDH release (Fig. 3B). If the neuroprotective effect of these drugs is due to acetylcholinesterase inhibition, it is difficult to explain why tacrine and rivastigmine did not decrease the LDH release induced by oxygen–glucose deprivation in this study, since they have relatively potent acetylcholinesterase-inhibitory activities (the IC_{50} values are 77 ± 1.4 and 4.3 ± 0.087 nM, respectively; Ogura et al., 2000). These results imply that the neuroprotective effect of donepezil may not be dependent on the acetylcholinesterase-inhibitory activity.

Experiments with a muscarinic receptor antagonist, scopolamine, and a nicotinic receptor antagonist, mecamylamine, showed that neither of them could antagonize the neuroprotective effect of donepezil (Fig. 4A and B). These results suggest that muscarinic and nicotinic activation induced by accumulated acetylcholine, which would result from acetylcholinesterase inhibition, is not responsible for the protective effect of donepezil. Thus, we concluded that the neuroprotective effect of donepezil does not depend on acetylcholinesterase inhibition.

However, donepezil is a selective acetylcholinesterase inhibitor, and no other mechanism(s) that could account for its neuroprotective action has been found thus far. It is difficult to speculate on what the mechanism might be. However, a relevant finding is that donepezil required a 12-h incubation time to exert a strong neuroprotective effect (Fig. 1). Responses that are produced via gene expression tend to involve a delay of this order. It was reported that huperzine A, another acetylcholinesterase inhibitor, attenuated H_2O_2 insult by modulating the expression of apoptosis-related genes in PC12 cells (Wang et al., 2001). Thus, the effect of donepezil observed in this study may also involve modulation of gene expression. However, further studies are needed to elucidate the actual mechanism(s) of the neuroprotective effect of donepezil. Such studies may provide a clue to a new target for dementia therapy.

In conclusion, the present findings demonstrate that donepezil has neuroprotective effects in an ischemia-induced neuronal cell injury model in vitro. This effect appears not to be based on acetylcholinesterase inhibition, but rather occurs via an unknown mechanism(s). Thus, we suspect that donepezil may alleviate cognitive impairment not only by improving cholinergic function in the brain, but also by inhibiting progressive neurodegeneration caused by cerebrovascular dysfunction in vascular dementia and Alzheimer's disease patients.

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