Full Paper

Donepezil Potentiates Nerve Growth Factor-Induced Neurite Outgrowth in PC12 Cells

Toru Oda¹, Toshiaki Kume¹, Hiroshi Katsuki¹, Tetsuhiro Niidome², Hachiro Sugimoto², and Akinori Akaike^{1,*}

Departments of ¹Pharmacology and ²Neuroscience for Drug Discovery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Received March 26, 2007; Accepted June 19, 2007

Abstract. Donepezil is a potent and selective acetylcholinesterase inhibitor developed for the treatment of Alzheimer's disease. To elucidate whether donepezil causes neuronal differentiation, we examined its effect on nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells. Donepezil (10 μ M) significantly potentiated the neurite outgrowth evoked by low (1 ng/ml) and high (50 ng/ml) concentrations of NGF. The effect of donepezil (1–10 μ M) was concentration-dependent. The enhancement of neurite outgrowth caused by donepezil was not blocked by the acetylcholine receptor (AChR) antagonists mecamylamine and scopolamine. Furthermore, the AChR agonists nicotine and carbachol did not affect the neurite outgrowth induced by NGF. Donepezil (10 μ M) also significantly potentiated neurite outgrowth evoked by dibutyryl cyclic AMP. Moreover, donepezil potentiated the NGF-induced phosphorylation of extracellular signal-regulated kinase (ERK). These results suggest that donepezil potentiated neuronal differentiation by enhancing the activation of ERK.

Keywords: donepezil, neurite outgrowth, nerve growth factor (NGF), PC12, extracellular signal-regulated kinase (ERK)

Introduction

Alzheimer's disease is a progressive neurodegenerative dementia characterized by impairments of memory and cognition. The main pathological findings in Alzheimer's disease are atrophy of the brain, amyloid deposition, and neurofibrillary degeneration (1, 2). Approximately 80% of the cholinergic neurons in the central nervous system are located in the nucleus basalis of Meynert, a structure providing major cholinergic pathways to the cortex. These neurons are known to undergo selective and severe degeneration in Alzheimer's disease (3). Neurobiochemical evidence additionally indicates a decrease in levels of choline acetyltransferase and a reduction in numbers of neuronal nicotinic acetylcholine receptors (AChRs) (4–6).

Central-type acetylcholinesterase (AChE) inhibitors, such as donepezil, galanthamine, tacrine, and rivastig-

mine, have been approved for the treatment of Alzheimer's disease because findings suggest that the activation of cholinergic systems is effective in improving the symptoms of the disease (7). However, distinct mechanisms would be involved in the therapeutic effects of donepezil (8), including its protective effect on glutamate-induced neurotoxicity via neuronal nicotinic AChRs such as α 4- and α 7-nicotinic AChRs (9, 10).

To determine whether donepezil has additional effects on nerve cells that could promote neuronal differentiation, we examined the ability of donepezil to induce the outgrowth of neurites in PC12 cells, a well-studied model of neuronal differentiation. In response to neurotrophic factors such as nerve growth factor (NGF), PC12 cells undergo a series of physiological changes culminating in a phenotype resembling that of sympathetic neurons (11). These changes involve various signaling pathways. An increase in intracellular second messengers such as cAMP induces neuronal outgrowth (12). In particular, activation of extracellular signal-regulated kinase (ERK), a mitogen-activated protein

*Corresponding author. aakaike@pharm.kyoto-u.ac.jp

Published online in J-STAGE: August 4, 2007

doi: 10.1254/jphs.FP0070563

kinase, has been shown to play critical roles in the outgrowth of neurites in PC12 cells (13).

In the present study, we investigated the effects of donepezil on neuronal differentiation using PC12 cells. We show here that donepezil promotes NGF- and dibutyryl cyclic AMP (dbcAMP)-induced neurite outgrowth. These results suggest that donepezil enhances neuronal differentiation in addition to the activation of cholinergic systems and neuroprotective effects.

Materials and Methods

Cell culture and reagents

PC12 cells were cultured (37°C, 5% CO₂) in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 10% horse serum (Invitrogen, Carlsbad, CA, USA), 4 mM glutamine (Nacalai Tesque, Kyoto), 100 U/ml penicillin (Meiji, Tokyo), and 100 µg/ml streptomycin (Meiji). Drugs and sources were as follows: nerve growth factor was purchased from Promega; poly-D-lysine, galanthamine-HBr, tacrine, physostigmine, carbamylcholine, mecamylamine-HCl, and (–)-scopolamine were from Sigma-Aldrich; (–)-nicotine tetrates were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, USA); and donepezil hydrochloride {(±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride; E2020} was supplied by Eisai Co., Ltd. (Tsukuba)

Quantification of neurite outgrowth

Twenty-four hours after plating, the cells were treated for 48 h with drugs in DMEM supplemented with 1% horse serum (differentiative condition) or 10% horse serum and 5% fetal bovine serum (proliferative condition). Neurite outgrowth was estimated by using phase-contrast microscopy and was quantified as the percentage of cells bearing neurite processes of >1 cell diameter in length.

Immunoblotting

Twenty-four hours after plating, the cells were treated for 5 min with drugs in DMEM supplemented with 1% horse serum and fixed. Cells were lysed in buffer containing Tris (20 mM), β-glycerophosphate (25 mM), EGTA (2 mM), Triton X-100 (1%), phenylmethylsulfonyl fluoride (1 mM), aprotinin (1%), dithiothreitol (2 mM), and vanadate (1 mM) on ice. Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Sample buffer [124 mM Tris, pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate (SDS), 4% 2-mercapto-ethanol, and

0.02% bromophenol blue] was added to the samples, which were then boiled and run on a 10% SDS-polyacrylamide gel. Separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) that was then blocked using 5% skim milk in 10 mM Tris-buffered saline containing 0.1% Tween 20 (TBS – T) at room temperature for >1 h and subsequently incubated with a mouse monoclonal phospho-ERK antibody (diluted 1:2,000; Cell Signalling Technology, Beverly, MA, USA) or a rat monoclonal ERK antibody (diluted 1:1,000, Cell Signalling Technology) overnight at 4°C. The membrane was then washed 3 times in TBS – T and incubated for 1 h at room temperature with a secondary anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase for visualization of specific band intensities with an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK) and exposed to Fuji X-ray film.

Statistics

Data were expressed as means ± S.E.M. The statistical significance of differences between groups was determined by a one-way analysis of variance (ANOVA) followed by Dunnett and Turkey's two-tailed test using InStat (Graph Pad Software). Statistical significance was defined as a probability value of less than 5%.

Results

Effects of donepezil on NGF-induced neurite outgrowth in PC12 cells

PC12 cells respond to NGF by beginning to extend processes similar to those produced by primary sympathetic neurons in culture (14). To determine the effect of NGF on neurite outgrowth, PC12 cells were treated with NGF (0.3 – 50 ng/ml) for 2 days. Neurite outgrowth was observed in a concentration-dependent manner and the number of cells with neurites was highest at 50 ng/ml (data not shown).

In order to investigate the effects of donepezil on neurite outgrowth in PC12 cells, donepezil was administered with NGF for 48 h. NGF (1 ng/ml) induced neurites to grow (Fig. 1Aa) and the addition of both NGF and donepezil increased the number of cells with neurites (Fig. 1Ab). Similarly, NGF (50 ng/ml) induced more cells to produce neurites (Fig. 1Ac) and the addition of donepezil also increased the number (Fig. 1Ad). Moreover, quantitative examination revealed that donepezil significantly potentiated the outgrowth of neurites induced by low (1 ng/ml) and high (50 ng/ml) concentrations of NGF (Fig. 1B). However, donepezil (10 µM) alone had no effect on neurite outgrowth (data

Fig. 1. Effect of donepezil on NGF-induced neurite outgrowth in PC12 cells. A: Typical microphotographs of the effect of donepezil on NGF-induced neurite outgrowth. PC12 cells were treated with a: NGF (1 ng/ml), b: NGF (1 ng/ml) plus donepezil (10 μM), c: NGF (50 ng/ml), or d: NGF (50 ng/ml) plus donepezil (10 μM) for 48 h. Bar = 50 μm. B: Effect of donepezil on neurite outgrowth. Cells were treated with NGF (1 ng/ml, 50 ng/ml) and donepezil (10 μM) for 48 h (n = 12). *** $P < 0.001$. C: Concentration-dependent effect of donepezil on NGF-induced neurite outgrowth. Cells were treated with NGF (1 ng/ml) and donepezil (0.1–10 μM) for 48 h (n = 12). * $P < 0.05$, ** $P < 0.01$, vs NGF (1 ng/ml).

Table 1. Effects of various acetylcholinesterase inhibitors on NGF-induced neurite outgrowth

AChE inhibitor	Control	10 μM	100 μM
Tacrine	29.3 ± 1.7	33.7 ± 2.3	49.5 ± 2.4**
Gаланthамine	29.3 ± 1.7	26.4 ± 2.8	32.6 ± 1.9
Physostigmine	37.4 ± 2.0	36.8 ± 1.6	34.8 ± 3.7

Cells were treated with NGF (1 ng/ml) and tacrine, galanthamine, or physostigmine for 48 h. ** $P < 0.01$, vs NGF (1 ng/ml).

not shown). Next, we investigated the concentration-dependency of the effect of donepezil (0.1–10 μM). Donepezil (1–10 μM) enhanced the outgrowth of neurites caused by NGF in a concentration-dependent manner (Fig. 1C).

Effects of other central-type AChE inhibitors on neurite outgrowth

We investigated the effects of central-type AChE inhibitors other than donepezil on the NGF-induced neurite outgrowth. As shown in Table 1, tacrine (100 μM) significantly potentiated the effect of NGF (1 ng/ml). On the other hand, galanthamine and physostigmine did not affect the NGF-induced outgrowth.

Involvement of AChRs in the promotional effects of donepezil on NGF-induced neurite outgrowth

Previously, we reported that donepezil protected

cortical neurons against glutamate-induced neurotoxicity via nicotinic AChRs (9, 10). To determine whether the activation of AChRs was involved in the effects of donepezil, we examined the effects of mecamylamine, a nicotinic AChR antagonist, and scopolamine, a muscarinic AChR antagonist, on the donepezil-induced enhancement of neurite outgrowth. Each antagonist was added 15 min prior to donepezil. The potentiation by donepezil was not affected by mecamylamine (10 μM) or scopolamine (10 μM) (Fig. 2A). These results indicate that the enhancement of NGF-induced neurite outgrowth by donepezil is not mediated via AChRs.

To confirm the results, we examined the effects of nicotine, a nicotinic AChR agonist, and carbachol, a nicotinic and muscarinic AChR agonist, on the outgrowth. Each agonist was added to NGF-containing medium. Nicotine and carbachol did not affect the NGF-induced outgrowth of neurites (Fig. 2: B and C). These

Fig. 2. Involvement of AChRs in the promotional effects of donepezil on NGF-induced neurite outgrowth. A: Effects of acetylcholine receptor antagonists on donepezil-induced potentiation of neurite outgrowth elicited by NGF in PC12 cells. Cells were treated with NGF (1 ng/ml), donepezil (10 μM), and mecamylamine (10 μM) or scopolamine (10 μM) for 48 h (n = 12). n.s.: not significant. B and C: Effects of acetylcholine-receptor agonists on neurite outgrowth in PC12 cells. Cells were treated with 10 μM carbachol (B) or 1–100 μM nicotine (C) for 48 h (n = 12).

Fig. 3. Effect of donepezil on dbcAMP-induced neurite outgrowth in PC12 cells. A: Typical micrographs of the effect of donepezil on dbcAMP-induced neurite outgrowth. PC12 cells were treated with a: medium, b: donepezil (10 μM), c: dbcAMP (1 mM), or d: dbcAMP (1 mM) plus donepezil (10 μM) under proliferative conditions for 48 h. Bar = 50 μm. B: Effects of donepezil on dbcAMP-induced neurite outgrowth (n = 12). ***P < 0.001.

Fig. 4. Effect of donepezil on NGF-induced ERK activation in PC12 cells. A: Representative data on the expression of p-ERK (upper panel) and ERK (lower panel) proteins following treatment with donepezil for 5 min detected by Western blotting. PC12 cells were treated with medium, NGF (50 ng/ml), NGF (50 ng/ml) plus donepezil (10 μM), or donepezil (10 μM). B: Effect of donepezil on NGF-induced ERK activation. PC12 cells were treated with medium, NGF (50 ng/ml), NGF (50 ng/ml) plus donepezil (10 μM), or donepezil (10 μM) for 5 min. Data were expressed as means ± S.E.M. for 5 experiments (n = 5). *P < 0.05.

results indicate that donepezil enhances the NGF-induced outgrowth without the involvement of AChRs.

Effects of donepezil on dbcAMP-induced neurite outgrowth

dbcAMP elicits neurite outgrowth in PC12 cells (12).

To investigate the effect of donepezil on neurite outgrowth, its influence on dbcAMP-induced outgrowth was examined. Neurite outgrowth was observed in cells treated with dbcAMP (1 mM) for 48 h (Fig. 3). Neurite outgrowth induced by dbcAMP (1 mM) was significantly potentiated by simultaneous treatment with

donepezil (10 μ M) (Fig. 3).

Effects of donepezil on NGF-induced ERK activation

Several studies indicate that the ERK cascade plays critical roles in both NGF-induced and dbcAMP-induced neurite outgrowth in PC12 cells (13). Therefore, donepezil could affect the phosphorylation of ERK. To examine the effect of donepezil on NGF-induced ERK activation, PC12 cells were treated with NGF and/or donepezil for 5 min. As shown in Fig. 4, NGF (50 ng/ml) induced ERK activation. When donepezil (10 μ M) was added with NGF (50 ng/ml), the effect was significantly potentiated.

Discussion

In the present study, we examined the effects of donepezil on neuronal differentiation. Donepezil potentiated both NGF- and dbcAMP-induced neurite outgrowth. Although it required a higher concentration than donepezil, tacrine also potentiated NGF-induced neurite outgrowth. On the other hand, galanthamine and physostigmine did not affect neurite outgrowth. Moreover, promotional effects of donepezil and tacrine on the NGF-induced neurite outgrowth were observed at concentrations much higher than the IC₅₀ values for AChE. These results indicate that the effect of donepezil to potentiate NGF-induced neurite outgrowth did not necessarily need AChE inhibitor activity.

Next, we investigated the mechanisms of the effects of donepezil. We previously reported that donepezil protected cortical neurons against glutamate-induced neurotoxicity via nicotinic AChRs (9, 10). Therefore, we examined the involvement of AChRs in the effect of donepezil. Mecamylamine and scopolamine did not influence the effect of donepezil. Moreover, nicotine and carbachol did not enhance NGF-induced neurite outgrowth. These results indicate that donepezil potentiates NGF-induced neurite outgrowth via AChR-independent mechanisms.

To determine whether donepezil has a promotional effect on neurite outgrowth induced by a stimulant other than NGF, we used dbcAMP. As shown in Fig. 3, donepezil also potentiated dbcAMP-induced neurite outgrowth. A previous study indicated that the ERK cascade plays critical roles in both NGF- and dbcAMP-induced neurite outgrowth in PC12 cells (13). Therefore, donepezil might augment neurite outgrowth by enhancing the phosphorylation of ERK. We examined the effects of donepezil on NGF-induced ERK activation. Donepezil potentiated the effect of NGF (Fig. 4). These results suggest that increasing the level of phospho-ERK could be one of the mechanisms by which

donepezil potentiates neurite outgrowth.

Among the AChE inhibitors, donepezil and tacrine potentiated NGF-induced neurite outgrowth. In addition, donepezil and tacrine have an affinity for σ -receptors (15). Takabayashi et al. reported that σ -receptor agonists enhanced NGF-induced neurite outgrowth (16). These previous findings suggest that σ -receptors are involved in the effect of donepezil to augment NGF-induced neurite outgrowth. The IC₅₀ value of donepezil against [³H]DTG binding to σ -receptors was reported to be 14.6 nM (15). On the other hand, as shown in Fig. 1C, an enhancing effect of donepezil on NGF-induced neurite outgrowth was observed at more than 1 μ M. This result suggests that effect of donepezil is not mediated via σ -receptors. We cannot exclude the possibility that donepezil would work at intracellular sigma receptors and a high concentration may be needed. However, σ -receptor agonists did not enhance dbcAMP-induced neurite outgrowth (16) and neurite outgrowth induced by dbcAMP was significantly potentiated by simultaneous treatment with donepezil (Fig. 3). Therefore, we assume that the outgrowth-enhancing effect of donepezil is not mediated via σ -receptors, but via other pathways. The precise mechanism of these effects of donepezil should be revealed by further studies.

In conclusion, it was shown that in addition to activating cholinergic systems and having a neuroprotective effect, donepezil potentiated NGF-induced neurite outgrowth via ERK activation in PC12 cells. The present findings suggest that donepezil could promote neuronal differentiation and this effect could promote the recovery of higher neuronal functions.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research and that on priority areas from the Ministry of Education, Culture, Science, Sports, and Technology, Japan. This study was also supported in part by the 21st Century COE program "Knowledge Information Infrastructure for Genome Science".

References

- 1 Yankner BA. A century of cognitive decline. *Nature*. 2000;404:125.
- 2 Selkoe DJ. The molecular pathology of Alzheimer's disease. *Neuron*. 1991;4:487-498.
- 3 Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science*. 1982;215:1237-1239.
- 4 Davies P, Maloney AJ. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet*. 1976;2:1403.
- 5 Guan ZZ, Zhang X, Ravid R, Nordberg A. Decreased protein

- levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. *J Neurochem.* 2000;74: 237–243.
- 6 Court J, Martin-Ruiz C, Piggott M, Spurdin D, Griffiths M, Perry E. Nicotinic receptor abnormalities in Alzheimer's disease. *Biol Psychiatry.* 2001;49:175–184.
  - 7 Wynn ZJ, Cummings JL. Cholinesterase inhibitor therapies and neuropsychiatric manifestation of Alzheimer's disease. *Dement Geriatr Cogn Disord.* 2004;17:100–108.
  - 8 Sugimoto H, Yamanishi Y, Iimura Y, Kawakami Y. Donepezil hydrochloride (E2020) and other cholinesterase inhibitors. *Curr Med Chem.* 2000;7:303–339.
  - 9 Takada Y, Yonezawa A, Kume T, Katsuki H, Kaneko S, Sugimoto H, et al. Nicotinic acetylcholine receptor-mediated neuroprotection by donepezil against glutamate neurotoxicity in rat cortical neurons. *J Pharmacol Exp Ther.* 2003;306:772–777.
  - 10 Takada-Takatori Y, Kume T, Sugimoto M, Katsuki H, Sugimoto H, Akaike A. Acetylcholinesterase inhibitors used in treatment of Alzheimer's disease prevent glutamate neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol 3-kinase cascade. *Neuropharmacology.* 2006;51:474–486.
  - 11 Keegan K, Halegoua S. Signal transduction pathways in neuronal differentiation. *Curr Opin Neurobiol.* 1993;3:14–19.
  - 12 Gunning PW, Letourneau PC, Landreth GE, Shooter EM. The action of nerve growth factor and dibutyryl adenosine cyclic 3':5'-monophosphate on rat pheochromocytoma reveals distinct stages in the mechanisms underlying neurite outgrowth. *J Neurosci.* 1981;1:1085–1095.
  - 13 Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell.* 1997;89:73–82.
  - 14 Green LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A.* 1976;73:2424–2428.
  - 15 Kato K, Hayako H, Ishihara Y, Marui S, Iwane M, Miyamoto M. TAK-147, an acetylcholinesterase inhibitor, increases choline acetyltransferase activity in cultured rat septal cholinergic neurons. *Neurosci Lett.* 1999;260:5–8.
  - 16 Takebayashi M, Hayashi T, Su TP. Nerve growth factor-induced neurite sprouting in PC12 cells involves sigma-1 receptors: implications for antidepressants. *J Pharmacol Exp Ther.* 2002;303:1227–1237.