Histological Protection by Donepezil Against Neurodegeneration Induced by Ischemia–Reperfusion in the Rat Retina

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Abstract. Although a blockade of acetylcholine esterase has been reported to suppress neuronal cell death induced by exogenous glutamate and β-amyloid, information is still limited regarding the neuroprotective effects of the acetylcholine esterase inhibitor donepezil. We histologically examined the effects of donepezil on neuronal injury induced by ischemia–reperfusion. Intravenous and intravitreous treatment with donepezil 15 min prior to ischemia dramatically reduced the retinal damage. The protective effect of donepezil in the ganglion cell layer was not affected by mecamylamine, a nicotinic acetylcholine-receptor antagonist, nor scopolamine, a muscarinic acetylcholine-receptor antagonist. The protective effect of donepezil in the inner plexiform layer was reduced not by mecamylamine, but by scopolamine. Neostigmine, a choline-esterase inhibitor, and pilocarpine, a muscarinic acetylcholine-receptor agonist, have protective effects in the inner plexiform layer and the inner nuclear layer. These results suggest that not only the activation of acetylcholine receptors but also a mechanism unrelated to acetylcholine-esterase inhibition contribute to the protective effect of donepezil on the ganglion cells in the ischemic–reperfused rat retina. Donepezil may be useful as a therapeutic drug against retinal diseases that cause neuronal cell death such as glaucoma with high intraocular pressure.

Keywords: donepezil, retina, ischemia, reperfusion, acetylcholine esterase

Introduction

Cell death of the retinal ganglion cells and the inner retinal neurons is a characteristic of retinal ischemia–reperfusion injury. It is believed that ischemia-induced injury plays an important role in some retinal diseases such as glaucoma, including open angle glaucoma, closed angle glaucoma, and normal-tension glaucoma, and central retinal vessel occlusions. The mechanism of the cell death induced by retinal ischemia is not completely understood and remains a very interesting area to explore. Endogenous substances such as glutamate, oxygen free radicals, nitric oxide (NO), and calcium are among the pathological causes of ischemia–reperfusion injury (1 – 3).

Glutamate is the principal excitatory neurotransmitter in the central nervous system, including the retina (4, 5). However, stimulation of some of the glutamate receptors by an excess amount of glutamate under pathologic conditions such as hypoxia (6) and ischemia–reperfusion (1) is toxic to neuronal cells. The activation of the N-methyl-D-aspartate (NMDA) receptor, a subtype of the glutamate receptor (7, 8), followed by excess Ca²⁺ influx via NMDA receptor–operated channels is thought to be involved in the predominant mechanism of neuronal excitotoxicity. In fact, excitotoxicity caused by the elevation of glutamate concentration in the retinal extracellular space near the glutamate-receptor channels is thought to be one of the mechanisms of neuronal cell death induced by glaucoma (9) and MK-801, an NMDA receptor–channel blocker, prevents retinal damage induced by retinal ischemia–reperfusion (10, 11).

Donepezil is an acetylcholine esterase inhibitor widely used for treatment of moderate Alzheimer’s disease and known to improve the cognitive performance and global function in the affected patients (12). Recently, donepezil has been reported to have neuroprotective effects against glutamate neurotoxicity, oxygen-glucose deprivation...
Both Alzheimer’s disease and glaucoma are chronic neurodegenerative diseases. Recent reports suggest that there are some similarities between Alzheimer’s disease and glaucoma, including activation of caspase and abnormal processing of amyloid precursor protein (15). Bayer et al. (16) reported that patients with Alzheimer’s disease and Parkinson’s disease had a larger risk of glaucoma. Recently, donepezil was reported to have a protective effect against retinal ganglion cell death induced by glutamate and axotomy (17).

In the present study, to clarify the potential utility of the drug for central retinal vessel occlusion and glaucoma, we assessed effects of donepezil on retinal ischemia–reperfusion injury in rats by comparing them with those of neostigmine, a choline-esterase inhibitor. In addition, to clarify the mechanism of the action of donepezil, the effects of inhibitors of acetylcholine receptors on the protective effects of donepezil and the effect of acetylcholine-receptor agonists on neurodegeneration induced by retinal ischemia–reperfusion were also tested.

**Materials and Methods**

**Animals**

In the present study, experimental procedures conformed to the Guiding Principles for the Care and Use of Laboratory Animals, approved by The Japanese Pharmacological Society. Male Sprague-Dawley rats weighing 230 – 300 g (Charles River Japan, Kanagawa) were anesthetized with pentobarbital sodium (50 mg/kg i.p., Nembutal® injection; Abbott Laboratories, North Chicago, IL, USA), and the rectal temperature of animals was maintained at 37°C during the experiment using a heating pad and heating lamp. The femoral veins were cannulated for administration of the drug, if necessary.

**Induction of retinal ischemia**

Induction of retinal ischemia was performed as previously described (18 – 21). Briefly, the anterior chamber of the one eye was cannulated with a 27-gauge needle connected to a bottle filled with saline. Retinal ischemia was induced by raising intraocular pressure to 130 mmHg by lifting the bottle for 60 min. The opposite eye of each animal served as a non-ischemic control.

**Drug injection**

Donepezil was administered intravenously and intravitreally. Intravenous injection was performed through a cannula indwelling in the femoral vein. Intravitreal injection was performed as previously described (10, 21). Briefly, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Injection was performed with a 33-gauge needle connected to a 25-μL gas-tight microsyringe (1702LT; Hamilton, Reno, NV, USA), which was inserted approximately 1-mm behind the corneal limbus. A 5-μL aliquot of drug solution, described below, was administered into both eyes. All drugs were dissolved with saline and administered 15 min before 60 min of retinal ischemia. When antagonists such as mecamylamine and scopolamine were used, we made a mixed solution with donepezil.

**Histological evaluation**

Histological evaluation was performed as previously described (18 – 21). Animals were euthanized by overdose of pentobarbital sodium 7 days after 60 min of ischemia and both eyes were enucleated. Enucleated eyes were fixed with Davidson solution, comprised of 37.5% ethanol, 9.3% paraformaldehyde, 12.5% acetic acid, and 3% glutaraldehyde for 1 – 12 h at room temperature. The fixed eye was bisected through the optic nerve head in the vertical meridian with a microtome blade (Histo Cutter Super #35 Type; Micro Glass, Tokyo) and embedded in paraffin after removing a lens. Five-micron horizontal sections through the optic nerve head of the eye were cut along the vertical meridian of the eye so as to contain the entire retina from the ora serrata in the superior hemisphere to the ora serrata in the inferior hemisphere using a microtome (HM325; Microm International, Walldorf, Germany) and a microtome blade (Histo Cutter Super #35 Type, Micro Glass). The sections were stained with hematoxylin and eosin and subject to morphometry. The sections showed oblique regions were excluded to avoid artifacts. The total number of the cells in the retinal ganglion cell layer (GCL) was counted for a length of 1 mm on either side of the optic nerve head beginning approximately 1 mm from the center of the optic nerve head in four independent sections under the direct observation of the HE-stained specimens using a light microscope (Optiphot-2; Nikon, Tokyo) and a counting machine. No attempt was made to distinguish the cell types in the GCL, and displaced amacrine cells were not excluded from the counts. Measurement of the thickness of the inner plexiform layer (IPL), the inner nuclear layer (INL), and the outer nuclear layer (ONL) was also performed to quantify the degree of cell loss induced by retinal ischemia–reperfusion. Digital photographs with approximately 0.25-mm width of the retinal layers in each section at a distance of approximately 1 mm from the center of the optic nerve head were taken using a digital camera (DP11; Olympus, Tokyo) connected to a light microscope. The photographs were printed onto A4 papers. Lines indicating the inner and outer borders of INL and ONL and the bottom of the ganglion cells were drawn on the printed photographs. To know the thicknesses of IPL, INL, and ONL, the distance between the
lines on the paper were measured. Averages for these measurements of thickness taken in five adjacent areas (approximately 40-μm intervals) were calculated. These parameters of each eye subjected to ischemia were normalized with those of the corresponding intact opposite eyes and are presented as percentages. We did all of the morphometrical analyses in a blind fashion.

Statistical analyses
The data represent the means ± S.E.M. One way analysis of variance followed by Tukey-Kramer test was used for multiple comparisons. Differences were considered to be statistically significant when the P-values were less than 0.05.

Results

Intravenous treatment with donepezil in the retinal ischemia–reperfusion model
First, we determined the effects of donepezil on the retinal injury induced by ischemia–reperfusion. Typical photomicrographs of the retina taken 7 days after reperfusion are shown in Fig. 1. In the vehicle-treated group, degenerative changes were observed in GCL, IPL, and INL of the ischemic eye, a characteristic of retinal ischemic atrophy (Fig. 1B), but such changes were not seen in the contralateral non-ischemic retina (Fig. 1A). Morphometric results at 7 days after reperfusion of 3–9 independent experiments are shown in Fig. 2. As indicated by the morphologic analysis, the IPL and INL in the ischemic–reperfused eye of the vehicle-treated group (n = 6) were thinner than in the contralateral normal eye at 7 days after reperfusion. No significant change was seen in the thickness of ONL after ischemia–reperfusion in all of the groups. Treatment with 3 mg/kg (n = 9) (Fig. 1F), but not 1 mg/kg (n = 3) (Fig. 1D), donepezil 15 min before 60 min of ischemia significantly reduced the amount of retinal damage. Intravenous treatment with donepezil itself did not affect the retinal morphology (Fig. 1: A, C, and E).

Fig. 1. Effect of intravenous treatment with donepezil on the histological damage induced by retinal ischemia–reperfusion. Representative photomicrographs showing histological appearance of the non-ischemic control (A, C, and E), and ischemic retinae 7 days after 60 min of ischemia (B, D, and F). Donepezil administered i.v. 15 min before 60 min of ischemia. Severe retinal damage is shown in the vehicle-treated (B) and 1 mg/kg donepezil–treated (D) retinae. In the 3 mg/kg donepezil–treated group, retinal damage is reduced (F). Scale bar = 50 μm. Original magnification is ×200.

Fig. 2. Effect of intravenous treatment with donepezil on the histological damage induced by retinal ischemia–reperfusion. Retinal damage was examined 7 days after 60 min of ischemia. The following 5 parameters of the ischemic eyes were normalized to those of the intact eyes (opposite side of the ischemic eye) and are presented as percentages: cell density in the GCL (ganglion cell layer) and thicknesses of the IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), and ONL (outer nuclear layer). The data represent the means ± S.E.M. *P < 0.05 vs. the vehicle-treated group.
Intravitreous treatment with donepezil and neostigmine in the retinal ischemia–reperfusion model

To verify whether acetylcholine esterase inhibition is involved in the protective effect of donepezil described above, we determined the effect of neostigmine, an acetylcholine esterase inhibitor on the retinal injury induced by ischemia–reperfusion. Because neostigmine is a quaternary ammonium compound and never passes through the retinal blood barrier, intravitreous injection was performed. Typical photomicrographs of the retina taken 7 days after reperfusion are shown in Fig. 3. In the vehicle-treated group, degenerative changes were seen in GCL, IPL, and INL of the ischemic eye, a characteristic of retinal ischemic atrophy (Fig. 3B), but such changes were not seen in the contralateral non-ischemic retina (Fig. 3A). No significant change was seen in the thickness of ONL after ischemia–reperfusion in all of the groups. Intravitreous treatment with 50 pmol/eye (Fig. 3F), but not 5 pmol/eye (Fig. 3D), donepezil 15 min before 60 min of ischemia markedly reduced the retinal degeneration. However, intravitreous treatment with 500 pmol/eye (Fig. 3J), but not 50 pmol/eye (Fig. 3H), neostigmine reduced the retinal damages only in IPL and INL. Intravitreous treatment with donepezil and neostigmine itself did not affect the retinal morphology (Fig. 3: A, C, and E). Morphometric results at seven days after reperfusion of five independent experiments are shown in Fig. 4.

Fig. 3. Effect of intravitreous treatment with donepezil and neostigmine on the histological damage induced by retinal ischemia–reperfusion. Representative photomicrographs showing histological appearance of the non-ischemic control (A, C, E, G, and I) and ischemic retinae 7 days after 60 min of ischemia (B, D, F, H, and J). Donepezil and neostigmine were injected intravitreously. Severe retinal damage is shown in the vehicle-treated (B) retinae. In the 50 pmol/eye donepezil–treated group (F), retinal damage is reduced. Neostigmine (500 pmol/eye) had protective effects in the IPL (inner plexiform layer) and the INL (inner nuclear layer) (J). Scale bar = 50 μm. Original magnification is ×200.

Fig. 4. Effect of intravitreous treatment with donepezil and neostigmine on the histological damage induced by retinal ischemia–reperfusion. Retinal damage was examined 7 days after 60 min of ischemia. The following 5 parameters of the ischemic eyes were normalized to those of the intact eyes (opposite side of the ischemic eye) and are presented as percentages: cell density in the GCL (ganglion cell layer) and thicknesses of the IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), and ONL (outer nuclear layer). The data represent the means ± S.E.M. *P < 0.05 vs. the vehicle group.
Effects of acetylcholine receptor antagonists on the protective effect of donepezil in the retinal ischemia-reperfusion model

To verify whether acetylcholine receptor stimulation is involved in the protective effect of donepezil described above, we determined the effects of mecamylamine, a nicotinic acetylcholine–receptor antagonist, and scopolamine, a muscarinic acetylcholine–receptor antagonist, on the retinal protection by donepezil against ischemia-reperfusion injury. Typical photomicrographs of the retina taken 7 days after reperfusion are shown in Fig. 5. Intra-

![Fig. 5. Effects of mecamylamine (50 pmol/eye) and scopolamine (15 pmol/eye) on the protective effects of donepezil in the ischemic–reperfused rat retina. Representative photomicrographs showing histological appearance of the non-ischemic control (A, C, E, and G) and ischemic retinae 7 days after 60 min of ischemia (B, D, F, and H). Donepezil (C, D, E, F, G, and H), mecamylamine (E and F), and scopolamine (G and H) were injected intravitreally. Scale bar = 50 μm. Original magnification is ×200.](image)

![Fig. 6. Effect of mecamylamine on the protective effects of donepezil in the ischemic–reperfused rat retina. Retinal damage was examined 7 days after 60 min of ischemia. The following 5 parameters of the ischemic eyes were normalized to those of the intact eyes (opposite side of the ischemic eye) and are presented as percentages: cell density in the GCL (ganglion cell layer) and thicknesses of the IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), and ONL (outer nuclear layer). Severe retinal damage is shown in the vehicle-treated retinae. In the 50 pmol/eye donepezil–treated group, retinal damage is reduced. Mecamylamine (50 pmol/eye) did not affect the protective effects of donepezil. The data represent the means ± S.E.M. *P < 0.05 vs. the vehicle group.](image)

![Fig. 7. Effect of scopolamine on the protective effects of donepezil in the ischemic–reperfused rat retina. Retinal damage was examined 7 days after 60 min of ischemia. The following 5 parameters of the ischemic eyes were normalized to those of the intact eyes (opposite side of the ischemic eye) and are presented as percentages: cell density in the GCL (ganglion cell layer) and thicknesses of the IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), and ONL (outer nuclear layer). Severe retinal damage is shown in the vehicle-treated retinae. In the 50 pmol/eye donepezil–treated group, retinal damage is reduced. Scopolamine (15 pmol/eye) reduced the protective effect in the IPL. The data represent the means ± S.E.M. *P < 0.05 vs. the vehicle group.](image)
vitreous treatment with mecamylamine (50 and 500 pmol/eye) did not affect the protective effect of donepezil (Fig. 5B). Intravitreous treatment with scopolamine (50 pmol/eye) reduced the retinal protection by donepezil only in IPL (Fig. 5C). Intravitreous treatment with mecamylamine and scopolamine itself did not affect the retinal morphology. Morphometric results at seven days after reperfusion of five independent experiments are shown in Figs. 6 and 7.

Intravitreous treatment with acetylcholine-receptor agonists in the retinal ischemia–reperfusion model

To verify whether acetylcholine-receptor stimulation protects against retinal ischemia–reperfusion injury, we determined the effects of nicotine and pilocarpine, a muscarinic acetylcholine–receptor agonist, on the retinal injury induced by ischemia–reperfusion. Typical photomicrographs of the retina taken 7 days after reperfusion are shown in Fig. 8. Intravitreous treatment with nicotine (50 pmol/eye) reduces the retinal damages in INL (Fig. 8B). Intravitreous treatment with pilocarpine (50 pmol/eye) reduces the retinal damages only in IPL and INL (Fig. 8C). Intravitreous treatment with nicotine and pilocarpine itself did not affect the retinal morphology. Morphometric results at seven days after reperfusion of five independent experiments are shown in Fig. 9.

Discussion

In the present study, we demonstrated that donepezil, an acetylcholine-esterase inhibitor, has a neuroprotective effect in the ischemic–reperfused rat retina for the first time. Donepezil was reported to increase acetylcholine content in the central nervous system (22). Acetylcholine activates two different types of acetylcholine receptors: nicotinic acetylcholine receptor and muscarinic acetylcholine receptor. Therefore, it is possible that donepezil protects retinal neurons against ischemia–reperfusion injury via activation of nicotinic acetylcholine receptors and/or muscarinic acetylcholine receptors. Actually, neuroprotection by activation of nicotinic acetylcholine receptors has been reported in various cultured neurons such as rat cortical neuron cultures (23), mixed retinal neuron cultures (24), and purified guinea-pig (25) and rat...
the vitreous body in a two-month-old rat is approximately 0.03 μL. Therefore, the maximum calculated intravitreous concentrations of donepezil, neostigmine, mecamylamine, scopolamine, nicotine, and pilocarpine are 3, 30, 30, 3, 3 and 3 μM, respectively. These concentrations of the drugs are enough to activate or block their targets. However, it is possible that low penetration of mecamylamine and scopolamine into INL causes the insufficient block of the protective effect of donepezil in INL. Because we are afraid that higher concentration of the drugs induces non-specific effects on the retina, especially on the ganglion cells, that is the nearest to the vitreous body and the most important drug target in the treatment of glaucoma, we did not try higher concentrations of the drugs in the present study. Further experiments are needed to clarify the underlying mechanisms more clearly.

In the present study, donepezil reduced the number of the apoptotic cells in the ischemic–reperfused rat retina. In the cultured rat retinal ganglion cells, HA 14-1, a bcl-2 inhibitor, was reported to reduce neuroprotection by donepezil against glutamate neurotoxicity (17). Therefore, it is possible that donepezil activates an anti-apoptotic cellular signal transduction pathway related to bcl-2.

In addition, donepezil has been reported to have various beneficial effects. First, donepezil was reported to reduce neuronal damage induced by β-amyloid not via NMDA-receptor blockade (14), suggesting that donepezil has a neuronal protective effect unrelated to NMDA-receptor blockade. Second, donepezil was reported to block NMDA receptors (34). The activation of NMDA receptor channel is reported to play an important role for ischemia–reperfusion injury in the rat retina (11). Therefore, NMDA-receptor blockade may possibly be involved in the mechanism of the neuroprotection by donepezil. However, other groups reported that donepezil failed to protect against glutamate-induced neurotoxicity in primary cultured rat cerebellar granule neurons (35) and that donepezil reduced neuronal damage induced by β-amyloid not via NMDA-receptor blockade (14). It is still unclear whether NMDA-receptor blockade is actually involved in the protective effect of donepezil or not.

Third, donepezil was reported to increase glutathione level and to reduce malondialdehyde level in the streptozotocin-induced dementia model rat (36). In addition, donepezil protected against cell injury induced by oxygen-glucose deprivation in the rat pheochromocytoma cells (13). These reports suggest that donepezil reduces the injury induced by oxidative stress. To sum up the reports described above, donepezil protects retinal neurons against ischemia–reperfusion injury via activation of various pathways, including elevating acetylcholine levels. However, further experiments are needed to clarify the underlying mechanisms.

Various chemicals such as MK-801, an NMDA-type glutamate-receptor antagonist, and l-NAME, an NO synthase inhibitor (11), have been reported to protect the retina against ischemia–reperfusion injury. However, these chemicals are not in practical use because of various problems, including concern about their safety. Donepezil is a commercially available medicine for the treatment of moderate Alzheimer’s disease, and it has been reported to cause very few serious systemic side effects (12, 37). It is reported that orally administered donepezil was absorbed rapidly and that the mean blood level of donepezil reached a peak at 30 minutes after oral administration in the rat (38). The reported rate of absorption of orally administered donepezil is over 95% in the rat (38). The reported bioavailability of orally administered donepezil is 56.1% in the rat (39). According to these previous data, not only intravenous but also oral donepezil will be effective. In fact, orally administered donepezil (12 mg/kg) was reported to reduce infarct size in the ischemic–reperfused rat brain (27). Therefore, donepezil has advantages over other new chemicals presently in development as medicines for treatment of retinal injury induced by high ocular pressure.
Unfortunately, treatment with donepezil at the end of 60 min of ischemia did not protect against the ischemic–reperfused retinal injury in the pilot study (data not shown). In our high-ocular-pressure model, no-flow complete ischemia is induced in the eyeball. Therefore, the drug injected after ischemia may reach the retina behind the critical time point for appearance of the protective effect. The typical ocular pressure of the glaucomatous patients with high ocular pressure is from 25 to 60 mmHg, suggesting that the patients keep some degree of blood flow in the retina. A low–blood-flow retinal ischemia model is needed to investigate the protective effect of the drug treated during a glaucomatous attack. As such an experimental model has not been reported previously, now we are trying to establish a low–blood-flow retinal ischemia model. After we establish this model system, we will try to investigate the protective effect of donepezil treatment during the induction of high ocular pressure.

In conclusion, donepezil, which is an acetylcholine-esterase inhibitor and widely used for treatment of moderate Alzheimer’s disease, reduced ischemic–reperfused injury in the rat retina. Stimulation of acetylcholine receptors is not likely to involved in the mechanism of the protective effect of donepezil in retinal ganglion cells, whereas its protective effect in IPL and INL may be partially caused by cholinergic stimulation. Although further studies are needed, the present study shows the possibility that donepezil is effective for preventing glaucomatous injury to retinal neurons.

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