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Cholinesterase Inhibitor Donepezil Dilates Cerebral Parenchymal Arterioles via the Activation of Neuronal Nitric Oxide Synthase

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Background: An acetylcholinesterase inhibitor donepezil currently is used to treat patients with Alzheimer disease. However, its direct effect on cerebral blood vessels has not been evaluated. The present study was designed to examine whether donepezil induces acute cerebral arteriolar dilation and whether neuronal nitric oxide synthase contributes to this vasodilator response.

Methods: Brain slices were obtained from neuronal nitric oxide synthase knock-out or C57BL/6J strain (control) mice as well as Wistar rats. Parenchymal arterioles were monitored using videomicroscopy. During constriction to prostaglandin $F_{2\alpha}$ (5×10^{-7} M), donepezil (10^{-9}–10^{-8} M) or acetylcholine (10^{-6}–10^{-4} M) was added. In some experiments, brain slices were treated with a nonselective or a selective nitric oxide synthase inhibitor (N^G-nitro-L-arginine methyl ester [10^{-4} M] and S-methyl-L-thiocitrulline [10^{-5} M], respectively). An immunohistochemical analysis was performed using antibodies for neuronal nitric oxide synthase and acetylcholinesterase.

Results: Acetylcholine concentration-dependently dilated rat parenchymal arterioles, while S-methyl-L-thiocitrulline as well as N^G-nitro-L-arginine methyl ester completely abolished this response. Donepezil produced arteriolar dilation, which was inhibited by S-methyl-L-thiocitrulline or N^G-nitro-L-arginine methyl ester. Donepezil failed to induce arteriolar dilation in the brain slice from the neuronal nitric oxide synthase knock-out mice. Immunohistochemical analysis revealed spatial relationship between neuronal nitric oxide synthase and acetylcholinesterase in the arteriolar wall.

Conclusions: Donepezil produces acute vasodilation induced by a selective activation of neuronal nitric oxide synthase in the cerebral parenchymal arterioles. This agent may be capable of enhancing this enzymatic activity directly or via acetylcholinesterase existing on the arteriolar wall.

ALZHEIMER disease, which is a leading cause of dementia in the elderly population, is well characterized by the impairment of central cholinergic neurotransmission.[1] Indeed, previous studies demonstrated the tight relationship between the cholinergic dysfunction in diverse brain areas and the severity of cognitive dysfunction.[2,3] Cerebral blood vessels reportedly are innervated with cholinergic nerve fibers in both humans and animals, resulting in augmentation of cerebral blood flow and/or vasodilation via activation of neuronal nitric oxide synthase.[4–8] Importantly, in patients with Alzheimer disease, cortical cerebral blood vessels and nitric oxide synthase–containing neurons are deprived of the cholinergic control, indicating such neurovascular deficits are major pathogenic factors underlying the malfunction of cerebral blood flow regulation in this diseased state.[9–12]

An acetylcholinesterase inhibitor, donepezil, is clinically used worldwide for patients with mild to severe Alzheimer disease.[1,13,14] Chronic treatment with donepezil is known to preserve regional cerebral blood flow in these patients.[15,16] However, there is no direct evidence showing the acute vasodilator effect of donepezil via nitric oxide synthase on cerebral blood vessels. In addition, recent studies have demonstrated that cholinergic activation provoked by oral donepezil reduces hypersensitivity of the spinal cord in an animal model of neuropathic pain, indicating that this agent may be a useful tool in pain medicine.[17,18] These results led us to the idea that studies evaluating the role as well as the mechanism of this compound in the modulation of neurovascular function will allow us to obtain crucial information for donepezil use in the clinical practice.

Therefore, the aim of this study was to examine in the brain whether clinically relevant concentrations of donepezil induce acute arteriolar dilation, and if so, whether this vasodilator response is mediated by the selective activation of neuronal nitric oxide synthase.

Materials and Methods

The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals in Wakayama Medical University (Wakayama, Japan). Male Wistar rats (300–400 g), neuronal nitric oxide knock-out mice or C57BL/6J mice as the control (40–55 g), were obtained from the Jackson Laboratory (Bar Harbor, ME) via Charles River Japan (Yokohama, Japan). Rats and mice were housed in groups of three in cages under standard conditions for animal studies (20–21°C, 12 h of artificial lighting/day), were fed *ad libitum*, and had free access

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to filtered tap water. Animals were anesthetized by inhalation of 3% halothane in 100% oxygen (3 l/min) to perform a midline thoracotomy, and 50 ml of saline was infused intracardially into the left ventricle while a right atrial incision simultaneously was made for blood drainage. The animals were then decapitated, and the brains were rapidly removed and rinsed with artificial cerebrospinal fluid of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, and glucose 11.

Brains were cut freehand into blocks containing the neocortex, followed by immediate sectioning into slices (125 mm thick) with a mechanical tissue slicer (Vibratomes 1000; Ted Pella, Redding, CA). Throughout the slicing procedure, brain blocks were continuously bathed in the perfusion solution bubbled with 95% O_2 and 5% CO_2 at 4°C. Individually, slices were then transferred to a recording chamber filled with the artificial cerebrospinal fluid, which was mounted on an inverted microscope (Olympus IX70; Shinjuku-ku, Tokyo, Japan). The recording system consisted of a recording chamber (3 ml) and a tubing compartment (7 ml) including the perfusion chamber. The slices were continuously superfused with perfusion fluid at the flow rate of 1.5 ml/min, bubbled with mixture gas of oxygen and carbon dioxide (P_{CO_2} = 40 mmHg, pH = 7.4, 37°C in the recording chamber). An intraparenchymal arteriole (4.0–8.0 μm in internal diameter, 11.0–16.5 μm in external diameter) was located within the neuronal tissue, and its internal diameter was continuously monitored with the live computerized videomicroscopy.[19] The videomicroscopy equipment consisted of an inverted microscope, a 40× objective (Olympus), and a 2.25× video projection lens (Olympus). The image of a parenchymal arteriole was transmitted to a video camera (C6790-81, Olympus) and displayed on a computer *via* a media converter (Physio-Tech; Chiyoda-ku, Tokyo, Japan).

The differentiation between the arteriole and the venule in the brain was based on previous studies documenting that, in the brain, a layer of smooth muscle cells should be identified in the arteriole and that the venule resembles a large capillary with no more to its walls than endothelial cells resting on a basal lamina.[20,21]

We calculated the ratio of internal to external diameter of the vessel, and a vessel demonstrating a ratio less than 0.5 was used as an arteriole for the following experiments. We defined the internal diameter as the length between the internal margins of arteriolar walls. Changes of internal diameter in cerebral microvessels were recorded on computer image files and then analyzed using the image analysis software with a sensitivity to 0.01 μm (Physio-Tech).[19,22] Microvessel diameters were derived as an average of four measurements taken along approximately 20 μm of vessel length.

Each slice was equilibrated for at least 30 min before the start of the experimental protocols. All experiments were performed during constriction in response to prostaglandin $F_{2\alpha}$ (5×10^{-7} M), a concentration that produces submaximal vasoconstriction of approximately 70% compared with maximal contraction induced by prostaglandin $F_{2\alpha}$ (10^{-5} M) in the cerebral parenchymal arterioles.[19] A nonselective nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (10^{-4} M) or a selective neuronal nitric oxide synthase inhibitor S-methyl-L-thiocitrulline (10^{-5} M) was applied 15 min before the addition of prostaglandin $F_{2\alpha}$ (5×10^{-7} M). Concentration-responses to donepezil (10^{-9}–10^{-8} M), acetylcholine (10^{-6}–10^{-4} M), or sodium nitroprusside (3×10^{-8} to 3×10^{-6} M) were obtained in a cumulative fashion. Only one concentration-response was made for each slice. The amount of dilation of the cerebral arteriole induced by vasodilators was normalized using the constriction produced by prostaglandin $F_{2\alpha}$ (5×10^{-7} M) in each arteriole. Therefore, the percent dilation was calculated by the following equation: % dilation = 100 × (the diameter after administration of the vasodilator minus the diameter after administration of prostaglandin $F_{2\alpha}$ [5×10^{-7} M]) / (the diameter of control condition minus the diameter after administration of prostaglandin $F_{2\alpha}$ [5×10^{-7} M]). Percentage of constriction to prostaglandin $F_{2\alpha}$ = 100 × (the diameter after administration of prostaglandin $F_{2\alpha}$ [5×10^{-7} M] minus the diameter of control condition) / the diameter of control condition.

Immunofluorescence Analysis

In some experiments, the brain slices were fixed with 10% formalin buffered with phosphate buffer solution (pH 7.2) and embedded in paraffin, followed by the preparation of 4-6–μm sections. After the deparaffinization, sections were incubated with phosphate buffer solution containing 1% normal donkey serum and 1% bovine serum albumin to reduce nonspecific reactions. The sections were further incubated with the combination of rabbit antineuronal nitric oxide synthase and goat antiacetylcholineesterase polyclonal antibodies at the concentration of 1 μg/ml at 4°C overnight. After incubation with Cy3-conjugated donkey antirabbit immunoglobulin G polyclonal antibodies and fluorescein isothiocyanate–conjugated donkey antigoat immunoglobulin G polyclonal antibodies (10 μg/ml) at room temperature for 30 min, the sections were observed by fluorescence microscopy and fluorescence images were digitally merged. As negative controls, specimens were incubated with normal rabbit and goat immunoglobulin G or in the absence of the primary antibodies.

Drugs

The following pharmacologic agents were used: acetylcholine, dimethyl sulfoxide, N^G-nitro-L-arginine methyl ester, S-methyl-L-thiocitrulline, sodium nitroprusside, and

prostaglandin $F_{2\alpha}$ (Sigma Aldrich, St. Louis, MO). The rabbit antineuronal nitric oxide synthase and goat antiacetylcholineesterase polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Donepezil was a generous gift from Eizai Pharmaceutical (Tokyo, Japan). Drugs except donepezil were dissolved in distilled water such that volumes of less than 60 μl were added to the perfusion system. The stock solution of donepezil (10^{-6} M) was prepared in dimethyl sulfoxide (3×10^{-4} M). Drugs were dissolved in distilled water such that volumes of less than 60 ml were added to the perfusion system. The concentrations of drugs are expressed as final molar concentrations.

Statistical Analysis

The data were expressed as means ± SD; n refers to the number of rats or mice from which brain slices were taken. Power calculations were done with the inhibitory effect of S-methyl-L-thiocitrulline on the vasodilation in response to donepezil as the primary end point, and we calculated that a sample size of five gave 80% power to detect a change of 16.1% in vasoconstriction at a significance level of 0.05 (SD = 8%). Statistical analysis was performed by StatView® 5.0 (SAS Institute, Cary, NC) or Sample Power® 2.0 (SSPS Japan, Tokyo, Japan). Data were evaluated using repeated-measures of analysis of variance or a factorial analysis of variance as appropriate, followed by Student-Newman-Keuls test as a *post hoc* analysis. Differences were considered to be statistically significant when $P < 0.05$.

Results

Figure 1 shows the representative example of the arteriole and the venule with similar external diameters (16.5 or 17.0 μm for the arteriole and the venule, respectively) in the brain parenchyma. The arteriole, which has a smooth muscle layer, has a smaller internal diameter (5.5 μm) compared with that of the venule (11.4 μm).

During submaximal constriction in response to prostaglandin $F_{2\alpha}$ (5×10^{-7} M), acetylcholine dilated rat parenchymal arterioles in a concentration-dependent fashion, while N^G-nitro-L-arginine methyl ester completely abolished this response (fig. 2A). Donepezil (3×10^{-9} and 10^{-8} M) produced arteriolar dilation, which was completely abolished by S-methyl-L-thiocitrulline as well as N^G-nitro-L-arginine methyl ester (fig. 2B).

Donepezil (3×10^{-9} and 10^{-8} M) induced cerebral arteriolar dilation in the brain slice from control mice (C57BL/6J strain mice), while it did not induce any dilator responses in that from the neuronal nitric oxide synthase gene knock-out mice (fig. 3A). In contrast, sodium nitroprusside similarly produced arteriolar dilation in the brain slices from both strains (fig. 3B).
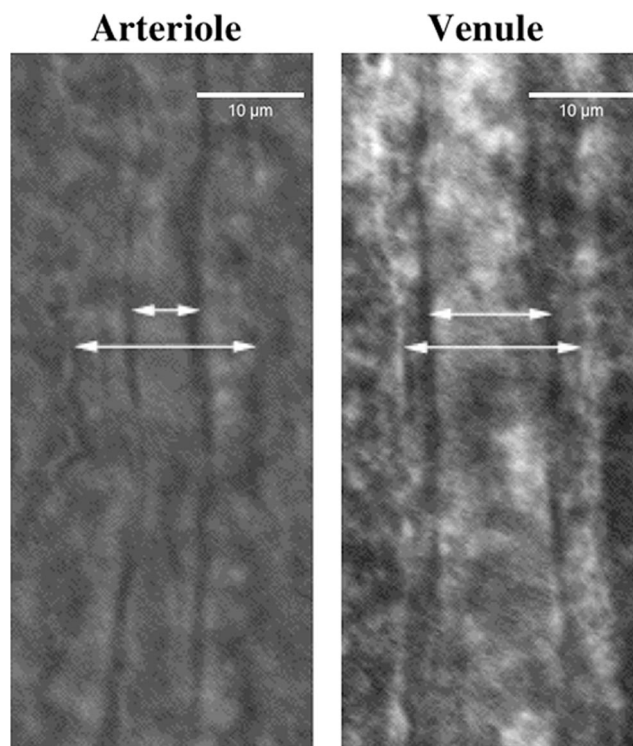

Fig. 1. The representative examples of an arteriole and a venule in the rat brain parenchyma. The arteriole, but not the venule, has a smooth muscle layer. The *longer* and *shorter arrow* in each figure demonstrate the external and the internal diameters, respectively.

A double-color immunofluorescence analysis revealed that in the brain parenchyma, neuronal nitric oxide synthase and acetylcholinesterase are similarly located in the arterioles as well as neuronal cells (fig. 4).

Discussion

Previous studies including ours suggest that cholinergic stimuli augment cerebral blood flow and/or vasodilation *via* activation of neuronal nitric oxide synthase.[6-8] These results are supported by studies showing that choline acetyltransferase–containing neurons project to neurons in which neuronal nitric oxide synthase is included, suggesting that augmentation of cholinergic neurotransmission contributes to increased cerebral blood flow mediated by neuronal nitric oxide synthase.[23] Histochemical evaluation also documented the existence of acetylcholinesterase in nerve fibers distributing to cerebral blood vessels, indicating the modulation of cerebral vasomotor function by the reduction of cholinergic neurotransmission.[4,5] In the current study, we have first revealed the spatial relationship between acetylcholinesterase and neuronal nitric oxide synthase in the brain structures, including neuronal cells and cerebral arterioles. Therefore, our results together with previous studies draw the conclusion that the modulation of cholinergic neurotransmission *via* changes in acetylcholine

Fig. 2. (*A*) Vasodilator responses to acetylcholine (10^{-6}–10^{-4} M) in the absence or in the presence of N^G-nitro-L-arginine methyl ester (10^{-4} M) in the rat cerebral parenchymal arterioles. (*B*) Vasodilator responses to donepezil (10^{-9}–10^{-8} M) in the absence or in the presence of N^G-nitro-L-arginine methyl ester (10^{-4} M) or S-methyl-L-thiocitrulline (10^{-5} M) in the rat cerebral parenchymal arterioles. Baseline internal diameter of arterioles were 7.0 ± 1.4 and 6.7 ± 1.5 μm for control arterioles and arterioles treated with N^G-nitro-L-arginine methyl ester for acetylcholine-induced vasodilation, and 6.2 ± 0.7, 5.9 ± 1.2, and 5.9 ± 0.3 μm for control arterioles, arterioles treated with N^G-nitro-L-arginine methyl ester, and S-methyl-L-thiocitrulline for donepezil-induced vasodilation, respectively. Data are shown as mean $\pm$ SD. Difference between the control arterioles and the arterioles treated with N^G-nitro-L-arginine methyl ester or S-methyl-L-thiocitrulline was statistically significant (* $P < 0.05$).
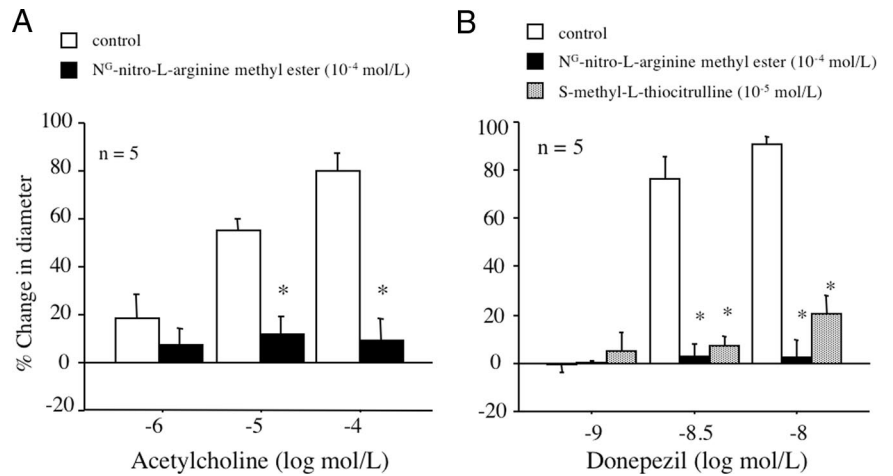

levels plays a role in the regulation of cerebral blood flow and/or vasodilation mediated by enzymatic activity of neuronal nitric oxide synthase.[24]

The impairment of cholinergic neurotransmission in the brain is reportedly one of the leading causes of cognitive dysfunction in patients with Alzheimer disease.[3,25,26] This is a reason why acetylcholinesterase inhibitors have been expected to ameliorate symptoms in these patients. Importantly, in patients with Alzheimer disease, cortical cerebral blood vessels and nitric oxide synthase–containing neurons are deprived of cholinergic control, indicating such neurovascular deficits are major pathogenic factors underlying the malfunction of cerebral blood flow regulation in this diseased state.[9-12] Therefore, it appears reasonable to administer acetylcholinesterase inhibitors to patients with Alzheimer disease to restore their cerebral blood flow. However, there has been no direct evidence showing vasodilator responses of cerebral arterioles to an acetylcholinesterase inhibitor, although *in vivo* studies using single-photon emission computed tomography documented that, in these patients, a highly selective acetylcholinesterase inhibitor donepezil enhances regional cerebral blood flow including that in the cerebral cortex.[1,27,28] In the current study, we have used brain slices from the cerebral cortex to evaluate the effects of donepezil, because the cortex receives cholinergic neurons and it is one of the most affected brain areas in Alzheimer disease.[25] We have found that donepezil induces cerebral parenchymal arteriolar dilation, which was completely abolished by a selective as well as a nonselective inhibitor of neuronal nitric oxide synthase.[29,30] More importantly, we have demonstrated that vasodilator responses to donepezil were absent in the parenchymal arterioles within the cerebral cortex slice from the neuronal nitric oxide synthase gene knock-out mice. Therefore, it is most likely that donepezil induces cerebral arteriolar dilation *via* the selective activation of neuronal nitric oxide synthase. In the current study, vasodilation in response to a nitric oxide donor sodium nitroprusside was not different between the gene knock-out and control mice, suggesting that the vasodilator response toward nitric oxide is preserved even in the condition with the neuronal nitric oxide synthase gene knock out.

The peak plasma concentration of donepezil in humans reportedly reaches 2–10 ng/ml after a single oral dose and the plasma protein binding is about 89%, indicating the plasma-free concentration of donepezil in

Fig. 3. Vasodilator responses to (*A*) donepezil (10^{-9}–10^{-8} M) and to (*B*) sodium nitroprusside (3×10^{-5} to 3×10^{-3} M) in the cerebral parenchymal arterioles of neuronal nitric oxide synthase knock-out and control mice. Baseline internal diameters of arterioles were (*A*) 4.2 ± 0.4 and 5.3 ± 0.8 μm and (*B*) 5.5 ± 0.7 and 4.8 ± 0.6 for control and neuronal nitric oxide synthase knock-out mice, respectively. Data are shown as mean $\pm$ SD. Difference between the arterioles of control and neuronal nitric oxide synthase knock-out mice was statistically significant (* $P < 0.05$).
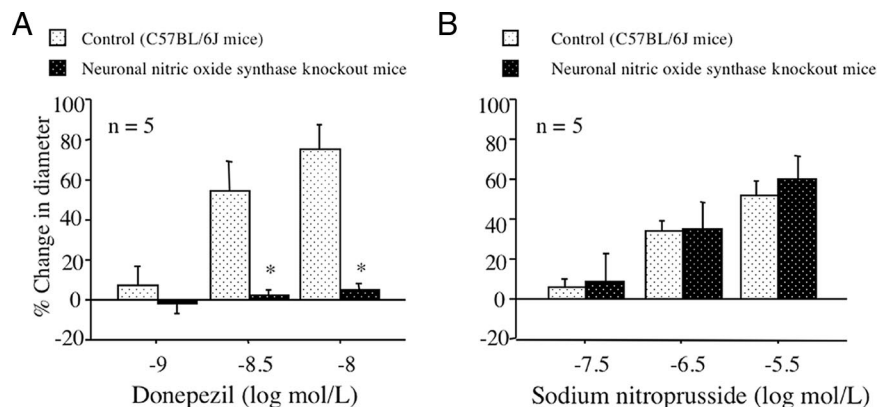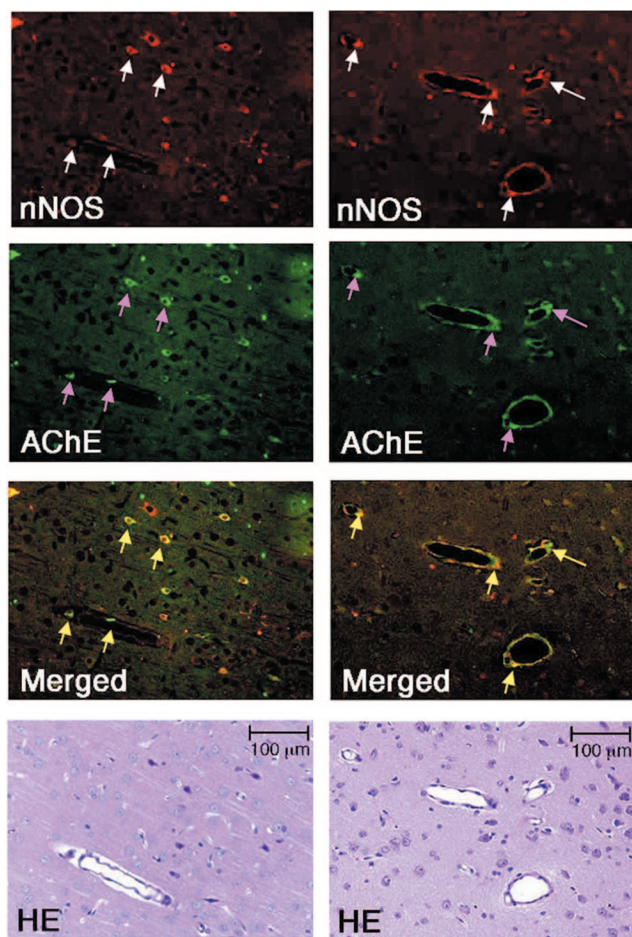
Fig. 4. A double-color immunohistochemical analysis for the rat brain slice containing the neocortex was performed to determine the localization of neuronal nitric oxide synthase (nNOS, Cy3 stain, *white arrows*), acetylcholinesterase (AChE, fluorescein isothiocyanate stain, *pink arrows*), and both (Merged, *yellow arrows*) as described in Materials and Methods. Hematoxylin and eosin (HE)–stained brain sections (at the *bottom*) were adjacent to those *upper* panels. Figure shows representative results from among five different rats, with *left* and *right panel* from each pair being from different rats. A scale bar indicates 100 μm in length in all pictures.

clinical practice would be up to 3×10^{-9} M (drug information supplied by Eizai Pharmaceutical, Tokyo, Japan). Therefore, concentrations of this agent used in the current study appear to be clinically relevant. We have documented spatial relationship between neuronal nitric oxide synthase and acetylcholinesterase in the cerebral parenchyma. Our results suggest the possibility that donepezil increases the local concentration of acetylcholine around cerebral parenchymal arterioles, leading to the dilation *via* the increased enzymatic activity of neuronal nitric oxide synthase. However, it is still unclear whether this agent is capable of enhancing the enzymatic activity directly or *via* acetylcho-

‡‡ Three way interaction between gabapentin, duloxetine, and donepezil in patients with diabetic neuropathy. Available at: http://clinicaltrials.gov/ct2/show/NCT00619983. Accessed April 8, 2008.

linesterase distributing to the cerebral arteriolar wall, and whether the enhanced neuronal activity induced by donepezil acts on the increase in cerebral blood flow.

It is important to note that in our experimental system, brain slices are maintained in artificial conditions in which vessels are not perfused and do not have intraluminal flow and that our confocal microscopic findings could be different from those in patients with Alzheimer disease. Therefore, it may be difficult to extrapolate directly our results to the *in vivo* diseased condition. However, as already pointed out by some researchers, it is also true that such preparations, in which neuronal–vascular interactions are preserved, offer an additional means to isolated microvessels and whole animal experiments for investigating the cerebral microcirculation.[31]

Donepezil is a leading product to treat patients with Alzheimer disease, as supported by clinical studies demonstrating the marked effectiveness of this agent on cognitive dysfunction.[1,32] The donepezil-induced cerebral arteriolar dilation shown in the current study may at least partly account for the clinical significance of this agent in Alzheimer disease. Donepezil reportedly prevents apoptotic neuronal death induced by glutamate neurotoxicity in the animal model, suggesting protective effects of this agent against cerebral damage such as ischemic brain injury.[33] In addition to the beneficial effects on the brain function, recent animal studies have demonstrated that cholinergic activation provoked by donepezil reduces hypersensitivity of the spinal cord in neuropathic pain, indicating that this agent may be useful to control such pain in humans.[17,18] More importantly, this acetylcholinesterase inhibitor is undergoing clinical trials in the treatment of diabetic neuropathic pain.‡‡ Therefore, the roles of donepezil in the regulation of the central nervous system should be increasingly crucial in the clinical practice including the perioperative period and pain medicine.

In conclusion, this is the first study demonstrating acute cerebral vasodilation induced by clinically relevant concentrations of donepezil. This compound appears to induce vasodilation *via* a selective activation of neuronal nitric oxide synthase. Our results provide a piece of information regarding the role of donepezil in the cerebral circulation.

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