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Acetylcholinesterase inhibitors attenuate angiogenesis

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ABSTRACT

Donepezil {(*RS*)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one} is a reversible acetylcholinesterase inhibitor and used for treatment of patients with AD (Alzheimer's disease). Recent studies showed that treatment with donepezil reduced production of inflammatory cytokines in PBMCs (peripheral blood mononuclear cells). It was also reported that muscle-derived inflammatory cytokines play a critical role in neovascularization in a hindlimb ischaemia model. We sought to determine whether donepezil affects angiogenesis. A hindlimb ischaemia model was created by unilateral femoral artery ligation. Blood flow recovery examined by laser Doppler perfusion imaging and capillary density by immunohistochemical staining of CD31-positive cells in the ischaemic hindlimb were significantly decreased in donepezil- and physostigmine-treated mice compared with control mice after 2 weeks. Donepezil reduced expression of IL (interleukin)-1β and VEGF (vascular endothelial growth factor) in the ischaemic hindlimb. Intramuscular injections of IL-1β to the ischaemic hindlimb reversed the donepezil-induced VEGF down-regulation and the anti-angiogenic effect. Hypoxia induced IL-1β expression in C2C12 myoblast cells, which was inhibited by pre-incubation with ACh (acetylcholine) or LY294002, a PI3K (phosphoinositide 3-kinase) inhibitor. Donepezil inhibited phosphorylation of Akt [also known as PKB (protein kinase B)], a downstream kinase of PI3K, in the ischaemic hindlimb. These findings suggest that cholinergic stimulation by acetylcholinesterase inhibitors suppresses angiogenesis through inhibition of PI3K-mediated IL-1β induction, which is followed by reduction of VEGF expression. Acetylcholinesterase inhibitor may be a novel anti-angiogenic therapy.

INTRODUCTION

Donepezil {(*RS*)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one} is a specific and reversible acetylcholinesterase inhibitor that increases bioavailability of ACh (acetylcholine), a neurotransmitter both in the CNS (central nervous system) and PNS (peripheral nervous system). Donepezil is used for

Key words: acetylcholinesterase inhibitor, angiogenesis, hindlimb ischaemia, interleukin-1β.

Abbreviations: ACh, acetylcholine; AD, Alzheimer's disease; bFGF, basic fibroblast growth factor; BP, blood pressure; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modified Eagle's medium; donepezil, (*RS*)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; HPF, high-power field; HR, heart rate; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; mAChR, muscarinic ACh receptor; MAPK, mitogen-activated protein kinase; nAChR, nicotinic ACh receptor; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cell; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; qRT–PCR, quantitative reverse transcription–PCR; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

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treatment of patients with AD and known to improve cognitive function [1]. Treatment of the patients with AD with donepezil is associated with reduction in the serum cytokine level and cytokine production from PBMCs (peripheral blood mononuclear cells) [2], suggesting a possible anti-inflammatory effect of donepezil.

Recent studies suggest that ACh, a neurotransmitter of the vagus nerve, has an anti-inflammatory effect [3]. VNS (vagus nerve stimulation) attenuated TNFα (tumour necrosis factor α) secretion from macrophages and hypotension induced by LPS (lipopolysaccharide) [4]. ACh inhibits activation of NF-κB (nuclear factor κB) [5] and induces suppressor of cytokine signal 3 expression in macrophages [6], resulting in the attenuation of inflammatory responses. Therefore it is possible that acetylcholinesterase inhibitor attenuates inflammation through activation of this so-called cholinergic anti-inflammatory pathway.

Inflammation is a regulated response to harmful stimuli such as infection and ischaemic/hypoxic injury [7] and also plays a pivotal role in neovascularization [8]. The enhancement of angiogenesis by inflammation is partly explained by production of various angiogenic growth factors such as VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), PDGF (platelet-derived growth factor) and MCP (monocyte chemoattractant protein)-1 from the leucocytes infiltrated into the ischaemic tissue [9]. It was also reported that IL (interleukin)-1β secreted from regenerating muscle after hindlimb ischaemia is critical for the enhanced neovascularization by implantation of PBMCs [10].

These studies prompted us to study the effect of cholinergic stimulation by donepezil on angiogenesis in a mouse model of hindlimb ischaemia. In the present study, we showed that pharmacological stimulation of cholinergic system by acetylcholinesterase inhibitors suppressed angiogenesis through inhibition of IL-1β induction.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium) was purchased from Gibco BRL. FBS (fetal bovine serum) was from JRH Biosciences. Donepezil was purchased from Tronto Research Chemicals. ACh, physostigmine, heat-inactivated horse serum and BSA were purchased from Sigma. Mouse recombinant IL-1β was purchased from PeproTec. An anti-CD31 (PECAM1) antibody was purchased from Santa Cruz Biotechnology. Antibodies against ERK (extracellular-signal-regulated kinase), p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase) and Akt and their phosphorylated (p) forms were obtained from Cell Signaling Technology. HRP (horseradish peroxidase)-conjugated secondary antibodies (anti-rabbit or anti-mouse IgG) were purchased from Vector Laboratories. SB203580 was a gift from GalxoSmithKline. PD98059 was purchased from BIOMOL Research Laboratories. LY294002 and SP600125 were purchased from Sigma. Other chemical reagents were purchased from Wako Pure Chemicals unless mentioned specifically.

Cell culture

C2C12 (mouse myoblast cell) cells were obtained from the RIKEN BioResource Center. C2C12 cells were maintained in low-glucose DMEM supplemented with 10% FBS (growth medium) at 37°C in a humidified atmosphere of 95% air/5% CO_2. C2C12 cells were grown to confluence, cultured in DMEM with 5% horse serum (differentiation medium) for additional 2 days and used in the experiment.

qRT–PCR (quantitative reverse transcription–PCR)

Total RNA was prepared by an acid guanidinium/phenol/chloroform extraction method with ISOGEN (Nippon Gene) according to the manufacturer's instructions. Then the total RNA (0.4 μg) was reverse-transcribed using moloney murine leukaemia virus RT (ReverTra Ace-α kit; Toyobo). The expression of mRNA was determined using SYBR-green (Toyobo) RT–PCR method and normalized to mouse β-actin expression. Primer sequences used in the present study are shown in Supplementary Table S1 (at http://www.clinsci.org/cs/123/cs1230241add.htm). ABI Prism 7500 Sequence Detection System (Applied Biosystems) was used.

Animal experiment

All procedures were approved by the institutional animal use and care committee and were conducted in conformity with institutional guidelines and Guide for Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). Nine-week-old C57BL/6 mice were purchased from Kyudo. Mice were anaesthetized by a bolus intraperitoneal injection of ketamine (90 mg/kg of body weight) and xylazine (4 mg/kg of body weight), which allowed approximately 60 min of anaesthesia for creation of hindlimb ischaemia and implantation of osmotic minipumps. The adequacy of anaesthesia was monitored by confirming the absence of the movement of the mice during the surgical procedure. To produce hindlimb ischaemia, the proximal portion of the left femoral artery including the deep branches was ligated, followed by ligation of distal portion of saphenous artery. The artery and all side branches between two ligation sites were excised completely. Then the mice were allotted to the donepezil group, the physostigmine group or the control group in a random manner. Donepezil was

suspended in water, and administered *ad libitum*. The estimated dose of orally ingested donepezil was 10 mg/kg of body weight per day. Some of the donepezil-treated mice were given an intramuscular injection of 0.5 ng of mouse recombinant IL-1β to the ischaemic limb on days 3, 5 and 7 after the operation.

Physostigmine (2 mg/kg of body weight per day) was administered via an osmotic minipump (Alzet). Physostigmine [11] and donepezil [12] at doses used in this study were reported to inhibit brain acetylcholinesterase activity by 62 and 45 % respectively. Nicotine (approximately 12 mg/kg of body weight per day) and bethanechol (approximately 20 mg/kg of body weight per day) were orally administered. The doses of nicotine and bethanechol were determined based on previous studies [13,14]. BP (blood pressure) and HR (heart rate) were measured using tail-cuff method (UR-5000; Ueda). Hindlimb blood perfusion was measured by a laser Doppler perfusion imaging system (Moor Instruments LDI). Before imaging, mice were placed on a heating plate at 37 °C to minimize the influence of temperature. After 2 weeks, mice were killed with an overdose of pentobarbital anaesthesia and the gastrocnemial muscle was quickly removed.

Immunohistochemistry

The gastrocnemial muscle was harvested and fixed overnight in 10 % buffered-formaldehyde. After fixation, the tissue was embedded in paraffin and serial cross-sections of the muscle were used for immunohistochemical analysis. Paraffin sections were deparaffinized and then subjected to heat-induced antigen retrieval for 15 min at 90 °C in citrate buffer (pH 6.0). Blocking of the section was performed with 2 % BSA for 1 h at room temperature (25 °C). The sections were incubated with a rat monoclonal anti-CD31 (PECAM1) antibody diluted 1:200 in blocking solution at 4 °C, which was followed by incubation with Alexa Fluor® 555-conjugated rabbit anti-(rat IgG) diluted 1:1000 in blocking solution. After incubation, the sections were rinsed in PBS three times. The slides were immersed in 70 % ethanol supplemented with 0.1 % Sudan Black B for 15 min and washed 70 % ethanol twice to eliminate or reduce autofluorescence as described previously [15]. The specimens were observed under a confocal microscope. An independent investigator blind to the treatment of the samples counted the number of positive cells.

Western blotting

Western blot analysis was performed by a conventional method as described previously [16]. Phosphorylated Akt was detected using a phospho-Akt antibody. The protein expression was detected by ECL® (enhanced chemiluminescence; GE Healthcare) according to the manufacturer's instructions. Membranes were scanned using LAS-4000mini (Fujifilm). The membranes were stripped and reprobed with an antibody against Akt (which recognizes both phosphorylated and non-phosphorylated forms of Akt) by the same procedure. The level of phosphorylated Akt was normalized to that of total Akt. Activation of ERK, JNK and p38 MAPK was examined by the same method. Physostigmine, an acetylcholinesterase inhibitor (10 nmol/l), was added to circumvent the effect of acetylcholinesterase in the culture medium for *in vitro* study.

ELISA

The serum of the mouse was collected and frozen at − 80 °C until assay. Concentrations of IL-1β, IL-6, TNFα and VEGF were determined by ELISA using appropriate commercial kits (R&D Systems). The gastrocnemial muscle of the ischaemic limb lysed in sample buffer of Western blot analysis was also subjected to ELISA for cytokine measurement.

Statistical analysis

Statistical analysis was performed with one-way ANOVA and Fisher test, if appropriate. Results are shown as means ± S.E.M. $P < 0.05$ was considered to be statistically significant.

RESULTS

Inhibition of angiogenesis by acetylcholinesterase inhibitors

Laser Doppler perfusion imaging revealed a rapid decrease and gradual recovery of the ischaemic/non-ischaemic hindlimb perfusion ratio after ligation of unilateral femoral artery (Figures 1A and 1B). The blood flow recovery of the hindlimb was significantly attenuated in donepezil-treated mice. Physostigmine, another acetylcholinesterase inhibitor that is structurally different from donepezil, also attenuated blood flow recovery, indicating that activation of cholinergic system attenuates angiogenesis in the hindlimb. Consistent with the results of laser Doppler perfusion analysis, treatment with donepezil or physostigmine significantly decreased the capillary density immunohistochemically evaluated with an anti-CD31 antibody in the ischaemic hindlimb (Figures 1C and 1D).

BP and body weight were not significantly different between acetylcholinesterase inhibitor-treated mice and control mice after 2 weeks of unilateral femoral artery ligation (see Supplementary Table S2 at http://www.clinsci.org/cs/123/cs1230241add.htm). Treatment with donepezil significantly reduced HR. However, physostigmine at the dose we used did not affect HR, indicating that HR reduction does not play a major role in the attenuation of blood flow recovery.

We then determined the effect of the specific agonist of mAChR (muscarinic ACh receptor; bethanechol) or

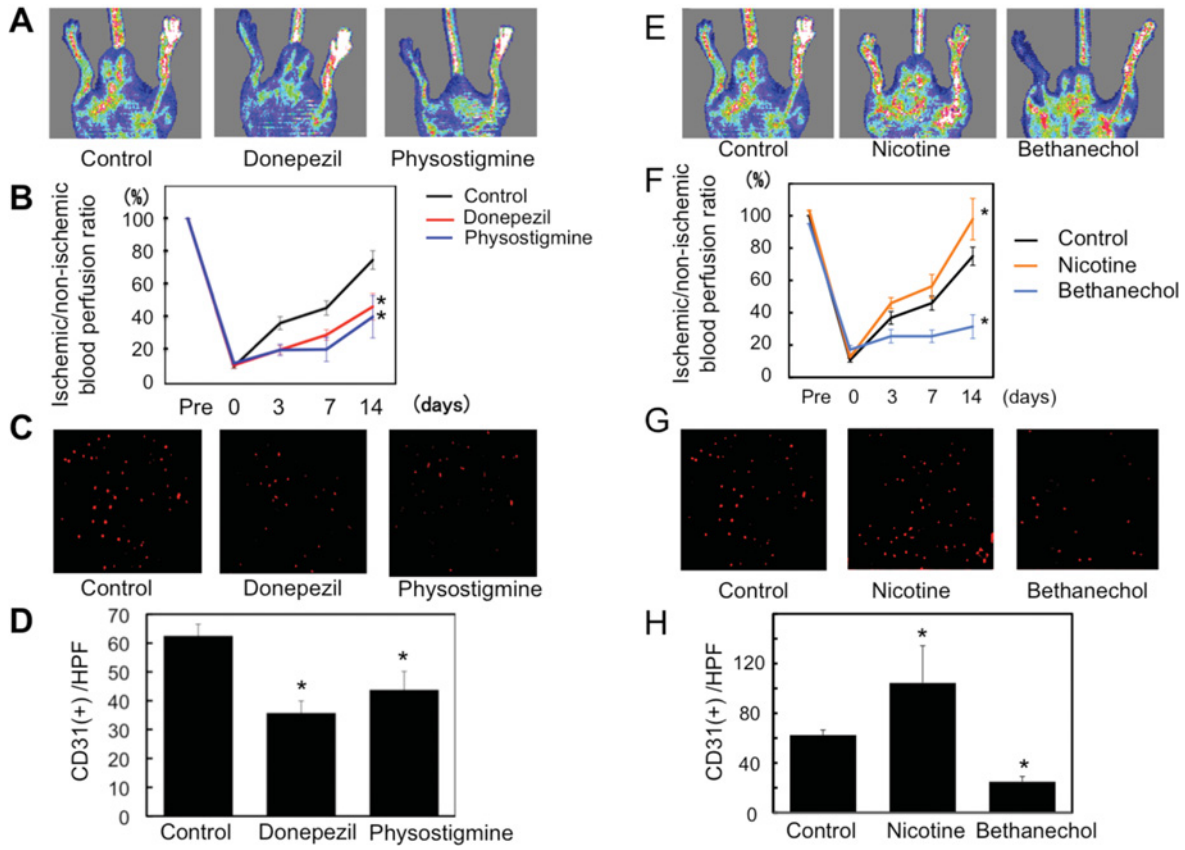

Figure 1 Acetylcholinesterase inhibitors attenuate neovascularization in a mouse model of hindlimb ischaemia

The effect of donepezil or physostigmine on blood flow recovery after hindlimb ischaemia was examined (A–D). The effect of bethanechol or nicotine on blood flow recovery after hindlimb ischaemia was examined (E–H). (A, E), Representative laser Doppler perfusion images of blood flow after 2 weeks of hindlimb ischaemia are shown. (B, F) Ratio of blood flow in the ischaemic hindlimb to that in the non-ischaemic hindlimb measured immediately (day 0) and at the days indicated in the Figure after unilateral femoral artery ligation. (C, G) Immunohistochemical staining for CD31 in the gastrocnemial muscle of ischaemic hindlimb is shown. (D, H) Capillary density expressed as the number of capillaries per HPF (high-power field). Results are expressed as means ± S.E.M. ($n = 7$). $^*P < 0.05$ compared with the control group.

nAChR (nicotinic ACh receptor; nicotine) on the blood flow recovery and capillary density of the ischaemic hindlimb (Figures 1E–1H). Bethanechol reduced blood flow recovery and capillary density of the ischaemic hindlimb, suggesting that mAChR is responsible for the attenuation of blood flow recovery by cholinergic stimulation. In contrast, nicotine enhanced blood flow recovery. Although the mechanism is not clear, the net effect of acetylcholinesterase inhibitors on angiogenesis favours mAChR stimulation in our model.

Donepezil suppressed VEGF and IL-1β expression in the ischaemic hindlimb

mRNA expression of angiogenic factors and cytokines in the ischaemic hindlimb harvested at day 7 was examined (Figure 2A). The expression of VEGF mRNA was significantly decreased in the donepezil-treated mice. However, expression of other angiogenic factors including bFGF, PDGF, PLGF (placental growth factor), Angpt (angiopoietin) 1 and 2 was not affected by donepezil treatment. Expression of IL-1β and TNFα mRNA in the ischaemic hindlimb was significantly decreased in the donepezil-treated group, suggesting an anti-inflammatory effect of donepezil. qRT–PCR also showed a trend towards reduction of CD3ε (T-lymphocytes) and F4/80 (macrophages) mRNA in the ischaemic hindlimb of the donepezil-treated mice compared with that of control mice. Although the difference was not statistically significant, the results suggest that treatment with donepezil mildly suppressed infiltration of inflammatory cells into the ischaemic hindlimb.

Protein level of IL-1β was significantly suppressed in an ischaemic hindlimb of donepezil-treated mice (Figure 2B). Although VEGF level was also decreased in an ischaemic hindlimb of donepezil-treated mice, the difference of VEGF level between control and donepezil-treated mice showed a borderline significance. We could not detect any changes in the serum levels of IL-1β, IL-6,

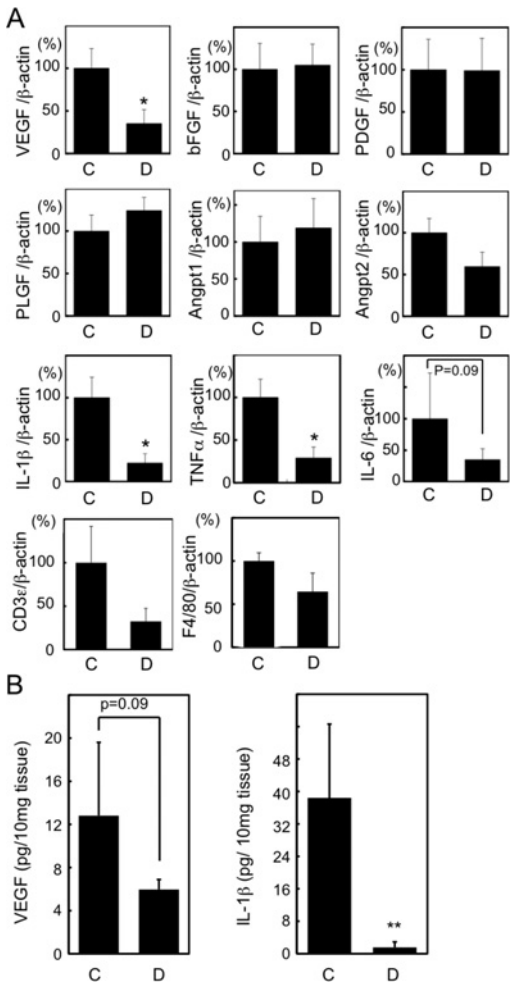

Figure 2 mRNA and protein expression in the ischaemic hindlimb of control and donepezil-treated mice

(A) mRNA expression of angiogenic factors and inflammatory cytokines in the ischaemic hindlimb of control (bar C) and donepezil-treated (bar D) mice was quantified with real time RT–PCR. The primer sequences used are indicated in Supplementary Table S1 at http://www.clinsci.org/cs/123/cs1230241add.htm. The expression level of each mRNA in the ischaemic hindlimb of control mouse was set as 100 % ($n = 7$). $^*P < 0.05$ compared with the control group. (B) Protein level of IL-1β and VEGF in the ischaemic hindlimb of control and donepezil-treated mice was measured by ELISA ($n = 4$). $^*P < 0.05$ compared with the control group.

or VEGF (results not shown). TNFα was not detectable in the serum and in ischaemic hindlimb in both groups. These findings suggest that donepezil locally suppressed inflammation in an ischaemic hindlimb.

IL-1β reversed the anti-angiogenic effect of donepezil

A previous report showed that recipient-derived IL-1β plays an important role in blood flow recovery in the ischaemic hindlimb [10,17]. As IL-1β expression was decreased in the ischaemic hindlimb of the donepezil-treated mice, we injected recombinant murine IL-1β into the ischaemic hindlimb. An injection of IL-1β restored the reduced blood flow recovery (Figures 3A and 3B) and capillary formation (Figures 3C and 3D) in the donepezil-treated mice. Donepezil-induced suppression of VEGF mRNA expression in the ischaemic hindlimb was also reversed by the IL-1β injection (Figure 3E). The IL-1β injection did not cause haemodynamic changes (see Supplementary Table S3 at http://www.clinsci.org/cs/123/cs1230241add.htm).

Role of PI3K (phosphoinositide 3-kinase) pathway in hypoxia-induced IL-1β induction

To gain an insight into the mechanism of donepezil inhibition of IL-1β expression, we used C2C12 cells, a mouse embryonic myoblast cell line. As hindlimb muscles are exposed to hypoxic conditions after femoral artery ligation, the effect of low oxygen concentration (1 % O_2) on IL-1β mRNA expression was determined. After exposure to the hypoxic condition for 12 h, IL-1β mRNA level was significantly increased compared with that in C2C12 cells incubated in normoxic (20 % O_2) conditions (Figure 4A). The induction of IL-1β by hypoxic conditions was significantly inhibited by pre-incubation with ACh. The Ach-induced suppression of IL-1β was reversed by the presence of atropine, a competitive antagonist of mAChR, but not by mecamylamine, a specific antagonist of nAChR. These results indicate that mAChR is responsible for the suppression of hypoxia-induced IL-1β expression. The findings are consistent with the *in vivo* results that a mAChR agonist bethanechol inhibited angiogenesis.

It was reported that PI3K and MAPKs are involved in IL-1β induction [18]. Treatment with LY294002 (PI3K inhibitor), PD98059 (ERK kinase inhibitor) or SP600125 (JNK inhibitor) significantly decreased hypoxia-induced IL-1β mRNA expression (Figure 4B). ACh attenuated hypoxia-induced phosphorylation of Akt, a downstream kinase of PI3K, but did not affect phosphorylation of ERK, JNK or p38 MAPK in C2C12 cells (Figures 4C–4F). These results suggest that ACh may suppress hypoxia-induced IL-1β expression through inhibition of the PI3K/Akt pathway. Indeed, the phosphorylation level of Akt but not other MAPKs was decreased in the ischaemic hindlimb of donepezil-treated mice (Figures 4G–4J).

DISCUSSION

We have shown in the present study that treatment with donepezil attenuated blood flow recovery of the ischaemic hindlimb in mice through reduction of the expression of IL-1β. It was suggested that mAChR is involved in this process. ACh as well as PI3K inhibitor suppressed hypoxia-induced IL-1β induction

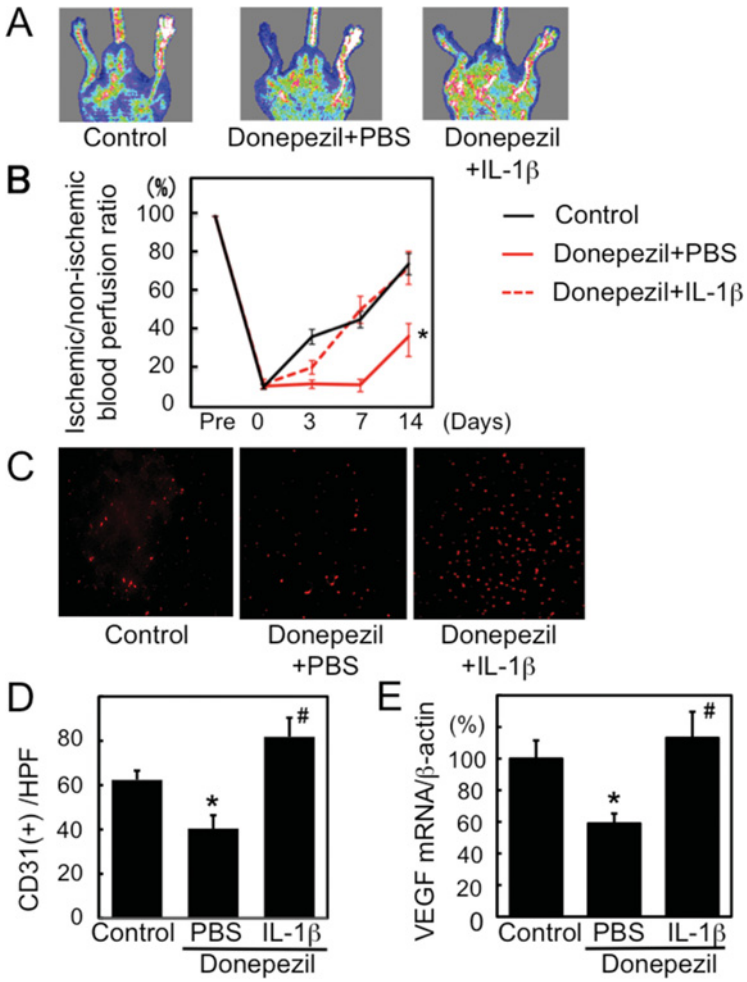

Figure 3 IL-1β reverses donepezil-induced inhibition of neovascularization

(A) Representative laser Doppler perfusion images of blood flow in the ischaemic hindlimb of donepezil-treated mice with or without IL-1β injection are shown. Control indicates ischaemic hindlimb without donepezil treatment. (B) Ratio of blood flow in the ischaemic hindlimb to that in the non-ischaemic hindlimb. $^{*}P < 0.05$ compared with control. (C) Immunohistochemical staining for CD31 in the gastrocnemial muscle of ischaemic hindlimb is shown. (D) Capillary density that is expressed as the number of capillaries per HPF. (E) mRNA expression of VEGF was quantified with real-time RT–PCR. Results are expressed as means $\pm$ S.E.M. ($n = 6-8$). $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with donepezil + PBS.

in C2C12 cells. Donepezil suppressed Akt activation in the ischaemic hindlimb, suggesting that an increase in Ach level by donepezil may inhibit hypoxia-induced IL-1β induction through suppression of the PI3K/Akt pathway, resulting in the inhibition of VEGF induction and angiogenesis.

A recent meta-analysis by Singh et al. [19] revealed that inhalation of anti-cholinergics is associated with a significant increase in the risk of cardiovascular events in patients with COPD (chronic obstructive pulmonary disease). Inhalation of anti-cholinergics significantly increased the risk of myocardial infarction and cardiovascular death without a statistically significant effect on the risk of stroke. This meta-analysis may support the idea that anti-cholinergics, contrary to acetylcholinesterase inhibitors, accelerate angiogenesis in the atherosclerotic plaque and thereby increase its vulnerability, which results in an increase in the cardiovascular events. However, a very recent double-blind trial that examined the effect of tiotropium, one of the anti-cholinergics, in patients with COPD showed opposite results [20]. Treatment with tiotropium showed an insignificant decrease in the number of death in patients with COPD and significantly decreased the incidence of myocardial infarction compared with placebo. Therefore the issue regarding the effect of anti-cholinergics treatment on cardiovascular events is still controversial.

Stimulation of nAChR is reported to enhance proliferation of endothelial cells and angiogenesis [13], which is consistent with the present study. Although stimulation of mAChR by bethanechol suppressed angiogenesis, the mechanism by which acetylcholinesterase inhibitors that

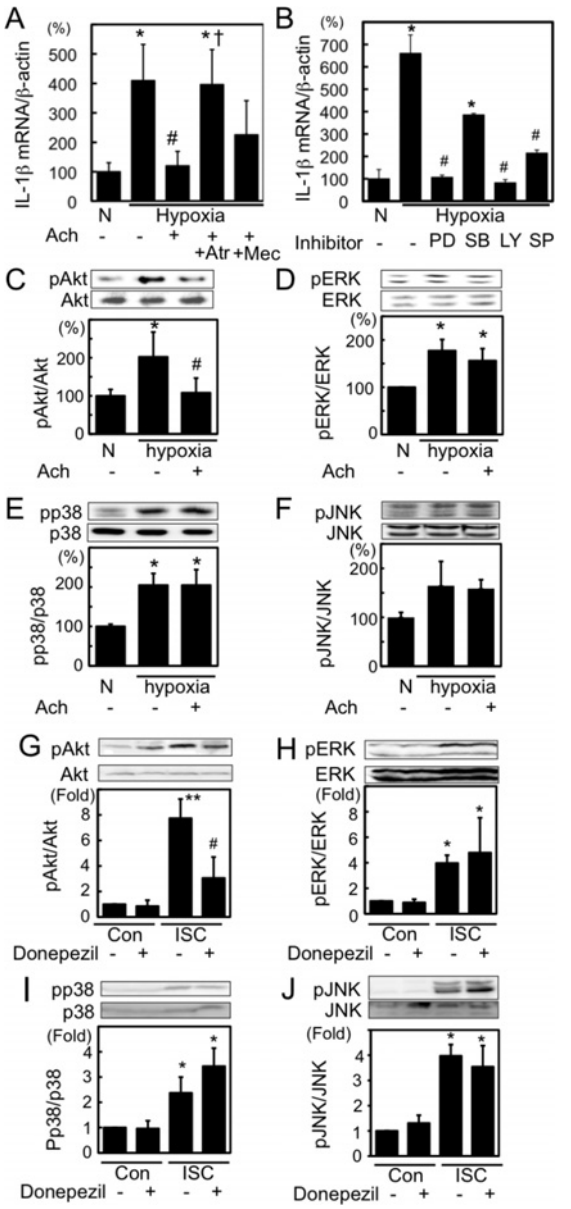

Figure 4 Ach and donepezil suppresses Akt activation

(A) C2C12 myoblast cells were pre-incubated with ACh (100 nmol/l) in the presence of atropine (Atr, 10 μmol/l) or mecamylamine (Mec, 10 μmol/l) prior to exposure to hypoxia (1 % O_2) for 1 h. After exposure to hypoxic condition for 12 h, the IL-1β mRNA level was determined with real time RT–PCR. N, normoxia. (B) The effects of PD98059 (PD; an ERK inhibitor, 10 μmol/l), SB203580 (SB; a p38 MAPK inhibitor, 10 μmol/l), LY294002 (LY; a PI3K inhibitor, 10 μmol/l) and SP600125 (SP; a JNK inhibitor, 10 μmol/l) on hypoxia-induced IL-1β mRNA expression in C2C12 cells were examined. mRNA expression of IL-1β in C2C12 cells cultured under normoxia (N) is used as control. $^{*}P < 0.05$ compared with under normoxic conditions; $^{\#}P < 0.05$ compared with hypoxia; $^{\dagger}P < 0.05$ compared with hypoxia + Ach ($n = 4$). (C–F) Effect of ACh on hypoxia-induced activation of Akt and MAPK was examined. (G–J) Effect of donepezil on the activation of Akt and MAPK in the non-ischaemic (Con) and ischaemic (ISC) hindlimb was examined. The ratio of phosphorylated form to total protein level of each kinase is shown in the histograms ($n = 4$). $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with the ischaemic hindlimb without donepezil.

are supposed to stimulate both nAChR and mAChR mimic the effect of mAChR is not clear.

Our *in vitro* results suggest that the increased ACh may be targeting ischaemic muscle of hindlimb, because Ach inhibited hypoxia-induced IL-1β expression in myoblast cells and donepezil reduced IL-1β expression in the ischaemic hindlimb. Therefore the anti-inflammatory effect of ACh on regenerating skeletal muscle may be dominant compared with direct effects of Ach on endothelial cells. Although we cannot exclude possible non-specific effects of these acetylcholinesterase inhibitors on angiogenesis, this is unlikely because the structure of donepezil and physostigmine is quite different.

The source of ACh in this hindlimb ischaemia model is not clear at this point. It is possible that an increase in ACh in the motor nerve ending of neuromuscular junction may play a role. Recent studies suggest that macrophages express choline acetyltransferase, which produces Ach from choline and acetyl-CoA [21]. Therefore infiltrated inflammatory cells may be another possible source of ACh. Alternatively, the ischaemic muscle itself may be the source of ACh, because it was previously reported that immunoreactivity of choline acetyltransferase is observed in both myoblasts and myotubes [22]. Another possibility is that acetylcholinesterase inhibitors may suppress angiogenesis in an indirect manner. mAChR in the CNS is reported to be involved in cholinergic anti-inflammatory pathway. Intracerebroventricular administration of muscarine, an agonist for mAChR, inhibited LPS-induced production of TNFα in the serum [23]. We cannot exclude the possible effect of these acetylcholinesterase inhibitors on the CNS in mediating an anti-angiogenic effect. Further study is needed to clarify the source and target cells of ACh in the ischaemic hindlimb.

A recent report showed that chronic hypoxia increased Akt phosphorylation in human macrophages [24]. Another report showed that TNFα-induced IL-1β expression is dependent on PI3K/Akt and NF-κB activation [18]. We showed that Ach suppressed hypoxia-induced IL-1β expression and Akt phosphorylation in C2C12 cells. And PI3K inhibitor suppressed hypoxia-induced IL-1β expression. Therefore it is suggested that Ach suppresses hypoxia-induced IL-1β expression through inhibition of PI3K/Akt pathway. Although it is known that PTEN (phosphatase and tensin homologue deleted on chromosome 10) negatively regulates PI3K/Akt pathway, we could not detect any change in PTEN expression in the ischaemic hindlimb in donepezil-treated mice (results not shown). The mechanism by which Ach inhibition of hypoxia-induced PI3K/Akt pathway is not clear and further study is needed.

The limitation of the present study is that the dose of donepezil used in this study is very high compared with

that clinically used for treatment of patients with AD. Therefore we must be cautious whether donepezil at a clinical dose affects angiogenesis in patients. A dose of 5–10 mg/kg of body weight per day of donepezil used in this study is widely used to examine the effect of donepezil on dementia in a rodent model [12] despite the fact that the clinical dose is 5–10 mg/day for patients with AD. It may be possible that differential susceptibility to the drug between humans and mice account for the requirement for high dose of donepezil in rodent models. A recent study showed a very small increase in skin temperature in the ischaemic hindlimb by donepezil, suggesting an angiogenic effect of donepezil [25]. The reason for the discrepancy between the previous study and our study is not clear at this point. However, the dose of donepezil administered to mice is higher in this study compared with the previous study (5 mg/kg of body weight per day), which may explain the discrepancy. Alternatively, the discrepancy may be because the previous report measured skin temperature rather than blood flow. In addition, the authors failed to examine the time course and measured surface temperature at later stage (28 days after ligation of femoral artery). We could not exclude the possibility that the difference of blood flow recovery of ischaemic hindlimb between control and donepezil-treated mice disappears or reverses after day 14 of femoral artery ligation. However, most of the study using C57BL/6 mice showed that blood flow recovery after hindlimb ischaemia reaches a plateau at day 14 or 21 [26,27]. Therefore this possibility is unlikely. And an anti-angiogenic effect of acetylcholinesterase inhibitor is confirmed by physostigmine in our study. Therefore it is suggested that acetylcholinesterase inhibitor has an anti-angiogenic effect at least under our experimental conditions.

In summary, we have shown in the present study that treatment with donepezil attenuated angiogenesis. Stimulation of cholinergic system may be a novel anti-angiogenic therapy.

AUTHOR CONTRIBUTION

Ryohei Miyazaki, Toshihiro Ichiki, Kotaro Takeda and Kenji Sunagawa contributed to the conception and design of the study, and writing of the paper. Ryohei Miyazaki performed the experiments. Jiro Ikeda, Aya Kamiharaguchi, Toru Hashimoto, Eriko Narabayashi and Hirohide Matsuura contributed to the *in vivo* experiments.

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SUPPLEMENTARY ONLINE DATA

Acetylcholinesterase inhibitors attenuate angiogenesis

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Table S1 Sequences of the primers used in real-time PCR

Angpt 1 and 2, angiopoietin 1 and 2; PlGF, placental growth factor.

	Sequence (5′→3′)	
Gene	Forward	Reverse
TNFα	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
VEGF	GCACATAGGAGAGATGAGCTTCC	CTCCGCTCTGAACAAGGCT
Angpt1	CCGAGCCTACTCACAGTACGACAG	AAATCGGCACCGTGTAAGATCAA
Angpt2	GGACAGTCATCCAACACCGAGA	CAAACTCATTGCCCAGCCAGTA
PDGF	CAAAGGCAAGCACCGAAAGTTTA	CCGAATCAGGCATCGAGACA
bFGF	GTGCCAACCGGTACCTTGCTA	TCAGTGCCACATACCAACTGGAG
PlGF	CCTGTCTGCTGGGAACAACTCA	CACCTCATCAGGGTATTCATCCAAG
CD3ε	CACTCTGGGCTTGCTGATGG	TCATAGTCTGGGTTGGGAACAGG
F4/80	GAGATTGTGGAAGCATCCGAGAC	GATGACTGTACCCACATGGCTGA
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATG

Table S2 HR, BP and body weight in the experimental groups

Results are means ± S.E.M. $^*P < 0.05$ compared with control.

Group	HR (beats/min)	SBP (mmHg)	Body weight (g)
Control	555 ± 34	101 ± 10	24.8 ± 0.8
Donepezil	452 ± 31*	97 ± 6	23.3 ± 0.3
Physostigmine	501 ± 20	100 ± 2	23.2 ± 0.7
Nicotine	522 ± 56	101 ± 17	24.3 ± 1.9
Betahnechol	472 ± 47	95 ± 8	23.8 ± 1.9

Table S3 HR, BP and body weight in the experimental groups treated with donepezil with or without IL-1β

Results are means ± S.E.M.

Group	HR (beats/min)	SBP (mmHg)	Body weight (g)
Control + PBS	531 ± 21	104 ± 6	24.1 ± 0.5
Donepezil + PBS	490 ± 26	100 ± 4	23.7 ± 1.7
Donepezil + IL-1β	501 ± 8	105 ± 3	23.6 ± 0.6

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