

# Arterial Baroreflex Dysfunction Impairs Ischemia-Induced Angiogenesis

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**Background**—Endothelium-derived acetylcholine (eACh) plays an important role in the regulation of vascular actions in response to hypoxia, whereas arterial baroreflex (ABR) dysfunction impairs the eACh system. We investigated the effects of ABR dysfunction on ischemia-induced angiogenesis in animal models of hindlimb ischemia with a special focus on eACh/nicotinic ACh receptor (nAChR) signaling activation.

**Methods and Results**—Male Sprague-Dawley rats were randomly assigned to 1 of 3 groups that received (1) sham operation (control group), (2) sinoaortic denervation (SAD)-induced ABR dysfunction (SAD group), or (3) SAD rats on diet with an acetylcholinesterase inhibitor pyridostigmine (30 mg/kg per day, SAD+Pyr group). After 4 weeks of the SAD intervention, unilateral limb ischemia was surgically induced in all animals. At postoperative day 14, SAD rats exhibited impaired angiogenic action (skin temperature and capillary density) and decreased angiogenic factor expressions (vascular endothelial growth factor [VEGF] and hypoxic inducible factor [HIF]-1 $\alpha$ ) in ischemic muscles. These changes were restored by acetylcholinesterase inhibition. Rats with ABR dysfunction had lower eACh levels than did control rats, and this effect was recovered in SAD+Pyr rats. In  $\alpha$ 7-nAChR knockout mice, pyridostigmine improved ischemia-induced angiogenic responses and increased the levels of VEGF and HIF-1 $\alpha$ . Moreover, nicotinic receptor blocker inhibited VEGF expression and VEGF receptor 2 phosphorylation (p-VEGFR2) induced by ACh analog.

**Conclusions**—Thus, ABR dysfunction appears to impair ischemia-induced angiogenesis through the reduction of eACh/ $\alpha$ 7-nAChR-dependent and -independent HIF-1 $\alpha$ /VEGF-VEGFR2 signaling activation. (*J Am Heart Assoc.* 2014;3:e000804 doi: 10.1161/JAHA.114.000804)

**Key Words:** acetylcholinesterase inhibitor • angiogenesis • arterial baroreflex • non-neural cholinergic system • peripheral vascular disease

Peripheral artery disease (PAD) is a growing health concern worldwide. Critical limb ischemia is a severe manifestation of PAD, characterized by intractable pain, ulcer, and gangrene. The clinical prognosis for patients with this

form of artery disease is poor, because up to one third of PAD patients are not amenable to conventional interventions, such as percutaneous angioplasty or surgical bypass, and they will ultimately require amputation of the limb.<sup>1</sup> Therapeutic neovascularization has been proposed as an alternative treatment in these “no”-option patients, and both growth cytokines and cells have shown impressive efficacy in the laboratory.<sup>2</sup> However, recent clinical trials of angiogenic therapies for ischemic disease have achieved only modest outcomes.<sup>3,4</sup> The mechanisms by which vascular angiogenic action is impaired in PAD are not yet well understood.

Arterial baroreflex (ABR) is an important mechanism in the regulation of cardiovascular activities.<sup>5</sup> It has been reported that decreased ABR sensitivity accelerates the progression of cardiovascular diseases (CVDs).<sup>6–9</sup> ABR dysfunction has been shown to promote atherosclerotic plaque growth<sup>10,11</sup> and it is indeed a target for treatment of atherosclerosis-related cerebral artery and coronary artery disease.<sup>12,13</sup> Recent evidence demonstrates that angiogenesis is essential in reestablishing blood supply to ischemic tissues and,

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consequently, to recovery of ischemic organ function in response to hypoxic injury.<sup>14–16</sup> These data suggested that impaired atherogenic vascular angiogenic action was associated with ABR dysfunction.

ABR deficiency has been proven to impair coronary vascular supply and reduce vascular growth in myocardium after myocardial infarction (MI).<sup>12</sup> The activation of vascular endothelial growth factor (VEGF)- and its receptor 2 (VEGFR2)-dependent angiogenic pathways represents a critical molecular mechanism by which ABR stimulation triggers angiogenesis.<sup>17</sup> Recently, several researches demonstrated that the non-neuronal cholinergic system is a critical modulator for angiogenesis.<sup>18–23</sup> Nicotine has been reported to stimulate angiogenesis as well.<sup>18,20</sup> We were curious about whether endothelium-derived acetylcholine (eACh) was involved in ischemia-induced angiogenic response and modulated by ABR dysfunctional status.

In addition, the function of  $\alpha 7$ -nicotinic ACh receptor ( $\alpha 7$ -nAChR) has been proven to be involved in proangiogenic signaling pathways, and pharmacological intervention-mediated ABR stimulation improves receptor signaling in infarcted myocardium and cultured vascular endothelial cells (ECs).<sup>19</sup> However, there is limited evidence as to whether expression of  $\alpha 7$ -nAChR in the lower extremities is also regulated by ABR sensitivity.

Accordingly, we investigated the role of ABR in ischemia-mediated angiogenesis with and without ABR dysfunction induced by sinoaortic denervation, with a special focus on vascular action associated with eACh/ $\alpha 7$ -nAChR-dependent and -independent VEGF/VEGFR2 signaling.

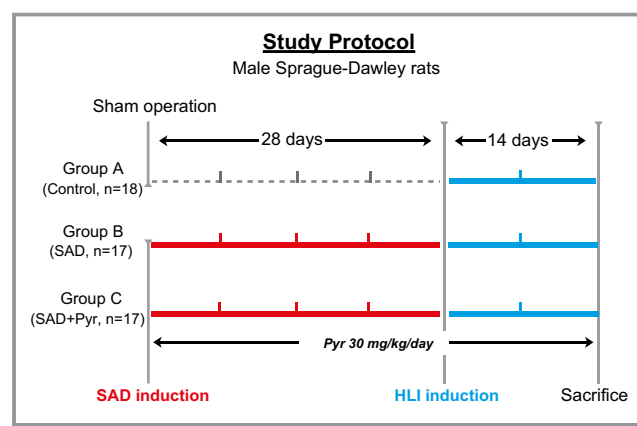
## Methods

### Animals

Male, 8-week-old Sprague-Dawley rats (250 to 300 g) and 10-week-old C57BL/6 mice (20 to 30 g) were obtained from the Sino-British SIPPR/BK Laboratory Animal Ltd. (Shanghai, China), whereas age-matched  $\alpha 7$ -nAChR knockout (KO; C57BL/6 background) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were housed with controlled temperature (22 to 25°C) and lighting (8:00 AM to 8:00 PM light and 8:00 PM to 8:00 AM dark) and had free access to tap water and standard rat chow. All animals used in this work received humane care in compliance with institutional guidelines of Shanghai Jiao Tong University (Shanghai, China) and Second Military Medical University (Shanghai, China).

### Study Protocol

Rats were randomly assigned to 1 of 3 groups and received either (1) a sham operation (control group,  $n=18$ ),



**Figure 1.** Study protocol and sampling time points. HLI indicates hindlimb ischemia; Pyr, pyridostigmine; SAD, sinoaortic denervation.

(2) sinoaortic denervation (SAD)-induced ABR dysfunction (SAD group,  $n=17$ ), or (3) SAD rats on the diet with an acetylcholinesterase inhibitor, pyridostigmine (Pyr; 30 mg/kg per day, SAD+Pyr group,  $n=17$ ; Selleck Chemicals, Houston, TX). At 4 weeks of the SAD intervention, unilateral hindlimb ischemia was surgically induced among all groups, as previously described.<sup>14</sup> A schematic diagram of the study protocol is shown in Figure 1. For mice experiments, all mice were subjected to unilateral femoral artery resection at day 0 to establish hindlimb ischemia<sup>16</sup> and fed by Pyr (30 mg/kg per day) until day 14. For the biological analysis, the animals were perfused with isotonic saline at physiological pressure after euthanization at an indicated time point after ischemic surgery. Thigh adductor muscles were then isolated and kept in liquid nitrogen (for the protein assay). For morphometry, after being immersed in fixative for 16 hours (4°C), muscles were embedded in paraffin.

### Animal Model of SAD

SAD has already been proven as an established baroreflex dysfunction animal model.<sup>10,12,13,24</sup> Rats were anesthetized with ketamine (50 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan), and carotid sinus baroreceptors were surgically denervated bilaterally to establish an experimental ABR dysfunctional model.

### Infrared Spectrum Imaging Analysis

The temperatures of both hindlimbs were measured by the infrared spectrum imaging analyzer (FLIR Systems, Wilsonville, OR). In this method, a color-coded image representing temperature distribution is displayed to evaluate blood perfusion. Low or no temperature is displayed as dark blue, and high temperature is displayed as red to white colors. At

every time point (before and after ischemic surgery at day 14), we performed 2 consecutive infrared spectrum scans over the same region of interest, and average perfusion values were computed from histograms of the colored pixels. To minimize variations resulting from ambient light, calculated temperature (relative units) is expressed as the ischemic/normal limb temperature ratio.

### Capillary Density

At postoperative day 14, capillary ECs in each cross-section (5  $\mu\text{m}$ ) were identified by immunohistochemistry with anti-CD31 monoclonal antibody (Ab; Becton Dickinson, Franklin Lakes, NJ). Capillaries were counted in 5 random microscopic fields ( $\times 400$  magnification) from 4 independent cross-sections of the adductor skeletal muscle in each animal. Capillary density is expressed as the number of capillaries per field.

### Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and cultured in endothelial basal medium 2 (EBM-2) plus 10% FBS and EGM-2 MV SingleQuotes (Clonetics) in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% air. HUVECs were seeded into 6-well plates at  $5 \times 10^5$  cells per well and cultured overnight in serum-free EBM-2. HUVECs at passage 5 to 8 were used. After washing twice with PBS, cells were treated with carbachol (Sigma-Aldrich, St. Louis, MO) and/or donepezil (Selleck Chemicals, Houston, TX) at indicated concentrations and time points. In some experiments, HUVECs were treated with a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002; Cell Signaling Technology, Beverly, MA) at indicated concentrations. Lysates and conditioned media were then collected for biological analysis.

### siRNA Transfection Protocol

Specific siRNA against hypoxic inducible factor 1 alpha (HIF-1 $\alpha$ ; 15345-001 F#569) was purchased from Sigma-Aldrich. HUVECs were grown on a 6-cm dish until they reached 30% confluence. The siRNA solution was mixed with Opti-MEM reduced-serum media containing Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA). The culture medium was then removed and switched to antibiotics-free EGM-2, together with 500  $\mu\text{L}$  of the transfection mixture, at a final concentration of siRNA in 100 nmol/L and were cultured for 48 hours.

### Western Blotting

Equal amounts of total protein from extracts of thigh muscle and HUVECs were resolved on SDS 10% PAGE and

transferred to nitrocellulose membranes for Western blotting with HIF-1 $\alpha$  (1:200 dilution), phospho-VEGFR2 (p-VEGFR2; Tyr996, 1:100 dilution), phosphor-protein kinase B (p-Akt; Ser473, 1:100 dilution), and  $\beta$ -actin (1:200 dilution; Cell Signaling Technology), as well as with choline acetyltransferase (ChAT; 1:100 dilution), vesicular acetylcholine transporter (VACHT; 1:100 dilution), and  $\alpha 7$ -nAChR (1:200 dilution; Sigma-Aldrich), followed by incubation with HRP-conjugated secondary Ab (GE Healthcare, Fairfield, CT), as described previously.<sup>16</sup> Positive signals were detected with an enhanced chemiluminescence (ECL) system (GE Healthcare). Relative protein levels were quantified using an ECL Western blotting detection kit (GE Healthcare) and ImageJ software (National Institutes of Health, Bethesda, MD).

### Real-Time Polymerase Chain Reaction

Total RNA was extracted from HUVECs lysates using TRIzol Reagent (Life Technologies, Carlsbad, CA) and were then reverse transcribed as previously described.<sup>16</sup> Quantitative gene expression was studied using the BIO-RED C1000 Real-Time Polymerase Chain Reaction (PCR) System with SYBR Green I agents (Life Technologies). Serial dilutions of a control sample of cDNA were used as the standard curve for each reaction. All experiments were performed in triplicate. The primers used (Sigma-Aldrich) were 5'-TACCCTACGGAGAGCGAAGA-3' and 5'-CTGTAGAGGCGAACATGACG-3' for VACHT; 5'-AAGACGCC-CATCCTGGAAAAG-3' and 5'-GTACGTGGCCAGGGTCTG-3' for ChAT; 5'-ATCACCATCTCCAGGAGCG-3' and 5'-CCTGCTTCAC-CACCTTCTTG-3' for GAPDH. Each RNA quantity was normalized to its respective GAPDH mRNA quantity.

### Enzyme-Linked Immunosorbent Assay

The concentration of VEGF, basic fibrotic growth factor (bFGF), and stromal-derived factor 1 (SDF-1) in the conditioned medium of cultured HUVECs were determined using ELISA kits according to the manufacturers' instructions (R&D Systems, San Diego, CA). The concentration of VEGF contained in ischemic tissue was measured with a kit purchased from Raybiotech (Norcross, GA).

### Immunofluorescence

After attachment of HUVECs (cell density at  $2 \times 10^4$  cells/well) to coverslips, cells were cultured in the presence or absence of donepezil (10  $\mu\text{mol/L}$ ) overnight. Then, cells were fixed for 10 minutes with 4% paraformaldehyde and washed with 1% glycerol. After perforation with 0.3% Triton X-100 (Sigma-Aldrich) and blocking with 5% BSA/PBS, cells were treated with 1  $\mu\text{g/mL}$  of mouse anti-ChAT or 3  $\mu\text{g/}$

mL of rabbit anti-human VAcHT (Sigma-Aldrich), respectively, overnight at 4°C. ChAT and VAcHT were visualized using goat anti-mouse (1:1000 dilution, Alexa 568; Life Technologies) or chicken anti-rabbit secondary Abs (1:1000 dilution, Alexa 488; Life Technologies), respectively. Coverslips were treated with VECTASHIELD mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and analyzed by confocal microscopy.

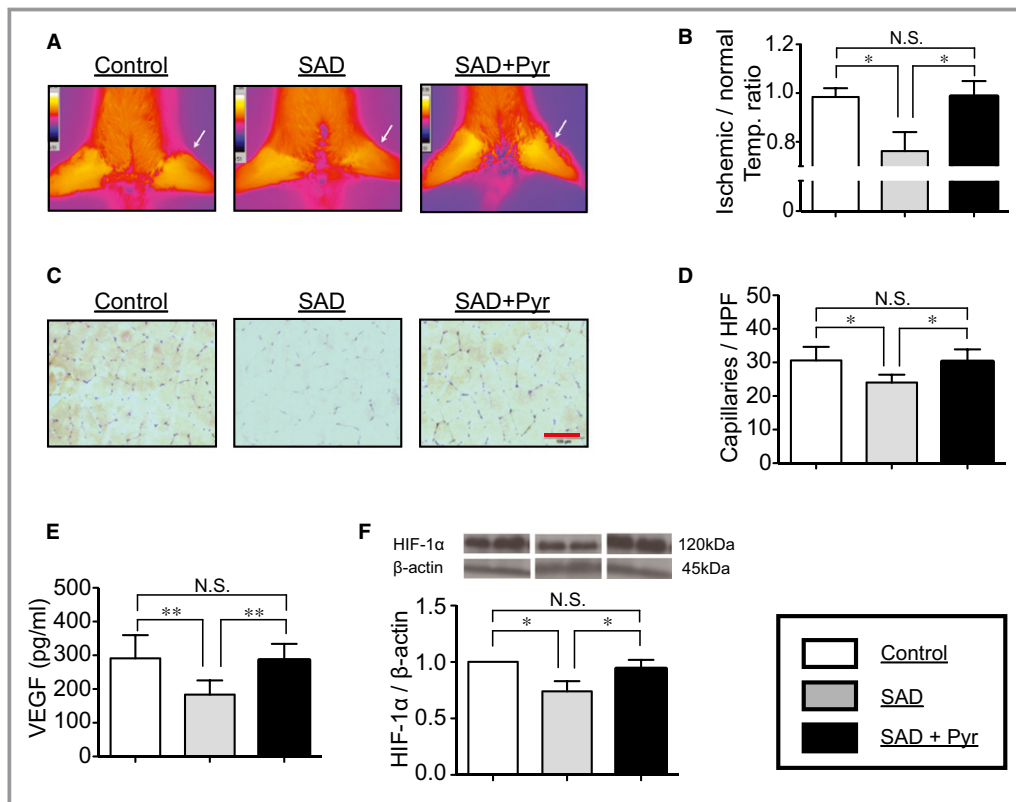
## Statistical Analysis

Data are expressed as means  $\pm$  SE. Student *t* tests (for comparisons between 2 groups) or one-way ANOVA (for 3 groups), followed by Tukey's post-hoc tests, were used for statistical analyses. SPSS software (version 17.0; SPSS, Inc., Chicago, IL) was used. A value of  $P < 0.05$  was considered significant.

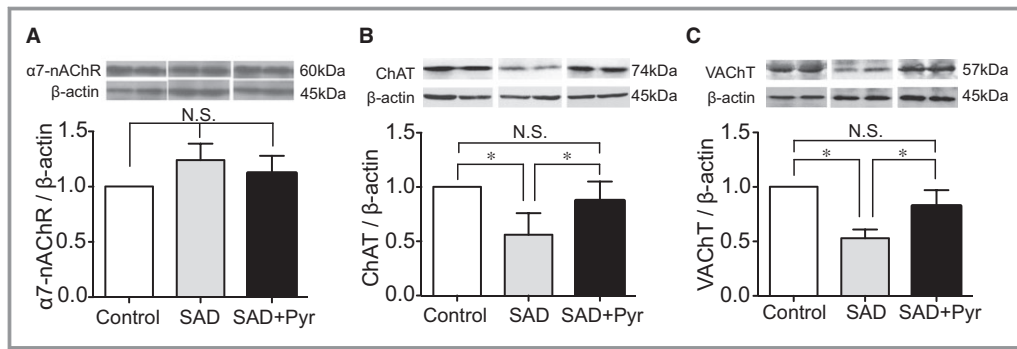
## Results

### Acetylcholinesterase Inhibition Improves ABR Dysfunction-Related Impaired Angiogenic Responses

As the first step in examining whether ABR dysfunction affects angiogenic actions in response to hypoxic injury, all rats were applied to hindlimb ischemia, and the ischemic/normal hindlimb temperature ratio and capillary density of ischemic muscles were evaluated. On day 14 after induction of ischemia, SAD rats exhibited a lower skin temperature ratio than controls, suggesting that ABR dysfunction delayed angiogenesis in response to hypoxia (Figure 2A and 2B). This was further supported by the quantitative data of capillary density, which indicated that capillary formation was impaired more in SAD rats than in control rats (Figure 2C and 2D). Furthermore, acetylcholinesterase inhibition by Pyr resulted in recovery of both skin temperature ratio and capillary formation (Figure 2A through 2D).



**Figure 2.** Hindlimb ischemia-induced angiogenesis in vivo. At day 14 after induction of ischemia, (A and B) infrared spectrum imaging assay, (C and D) anti-CD31 staining, (E) ELISA to the content of vascular endothelial growth factor (VEGF), and (F) Western blotting assay to expression of hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) in the ischemic area were performed to evaluate angiogenic actions. Infrared spectrum imaging data were analyzed and illustrated as an ischemic/normal hindlimb temperature ratio. Data of Western blotting are represented as fold of control.  $n=6$  in each group. \* $P < 0.05$ , \*\* $P < 0.01$ ; N.S., no significant difference; Tukey's post-hoc test. Temp, temperature. Scale bar: 50  $\mu$ m. HPF indicates high power field; Pyr, pyridostigmine; SAD, sinoaortic denervation.



**Figure 3.** Acetylcholine (ACh)-related protein expression in vivo. Protein expressions of (A)  $\alpha 7$ -nicotinic acetylcholine receptor ( $\alpha 7$ -nAChR), (B) choline acetyltransferase (ChAT), and (C) vesicular acetylcholine transporter (VACHT) in the ischemic area were determined among 3 experimental groups. Data of Western blotting are represented as fold of control.  $n=6$  in each group.  $*P<0.05$ ; N.S., no significant difference; Tukey's post-hoc test. Pyr indicates pyridostigmine; SAD, sinoaortic denervation.

To further examine the mechanism underlying the impairment of ischemia-induced angiogenic action in rats with ABR dysfunction, we evaluated levels of VEGF and HIF-1 $\alpha$  protein expressions in local tissue among the 3 groups of rats at day 14 after induction of ischemia. Protein levels of VEGF and HIF-1 $\alpha$  were both significantly lower in SAD rats than in control rats (Figure 2E and 2F). Pyr restored not only tissue VEGF concentration, but also HIF-1 $\alpha$  expression in ischemic tissue of SAD rats (Figure 2E and 2F). There were no differences of  $\alpha 7$ -nAChR expressions in ischemic muscle of rats among the 3 experimental groups (Figure 3A).

### Effects of Acetylcholinesterase Inhibition on ChAT and VACHT Expressions In Vivo and In Vitro

In order to test whether the endogenous ACh system was affected under the condition of dysfunctional ABR status, we checked 2 key components involved in synthesis and secretion of endogenous ACh after hindlimb ischemia. Protein expressions of ChAT and VACHT declined in SAD rats, compared to controls; however, Pyr magnified expressions of ChAT and VACHT, even under ABR dysfunctional status (Figure 3B and 3C). In vitro, expressions of ChAT and VACHT genes and proteins in cultured HUVECs were analyzed by real-time PCR, Western blotting, and immunofluorescence. We observed that both targeted genes and protein expressions were enhanced in HUVECs when exposed to donepezil (Figure 4A through 4F).

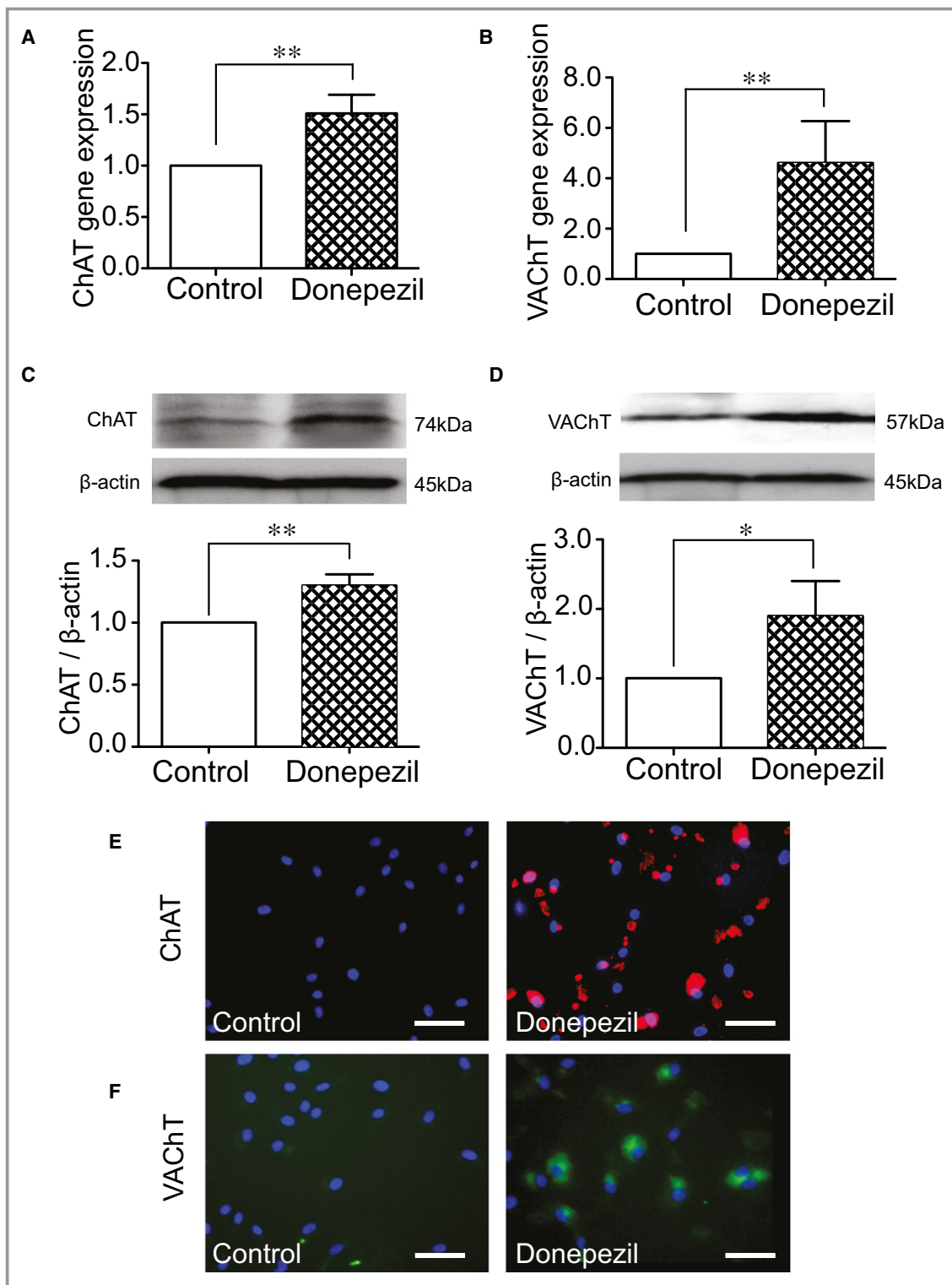
### Effects of Donepezil-Mediated Acetylcholinesterase Inhibition on Expression of Angiogenic Factors In Vitro

After treatment with donepezil for 24 hours, the conditioned medium and lysates of HUVECs were collected,

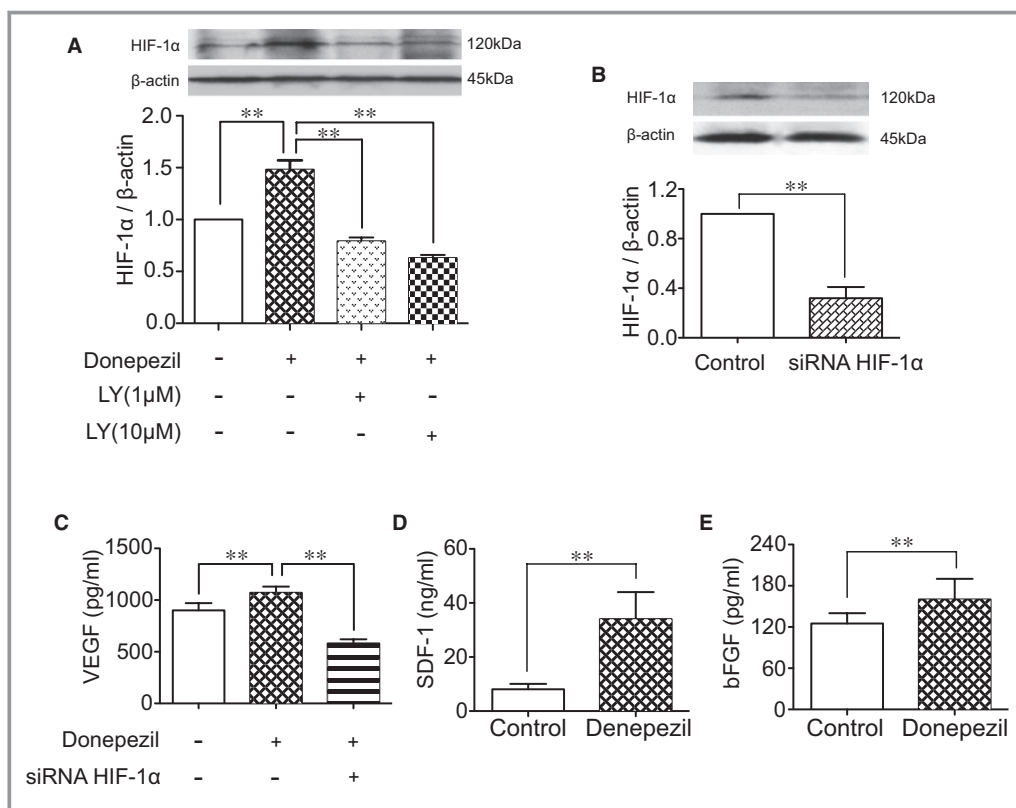
and several angiogenic factors were quantified using ELISA and Western blotting. Donepezil treatment resulted in increased levels not only of HIF-1 $\alpha$  protein in lysates (Figure 5A), but also VEGF, bFGF, and SDF-1 proteins in the conditioned medium (Figure 5C through 5E). Next, HUVECs were treated with a PI3K inhibitor (LY294002). LY294002 reduced donepezil-mediated HIF-1 $\alpha$  expression (Figure 5A). Moreover, HIF-1 $\alpha$ /RNA interference decreased the concentration of VEGF in the HUVEC conditioned medium after donepezil treatment (Figure 5B and 5C). These results indicate that autocrine secretion of eACh stimulates the angiogenic signal pathway of ECs.

### Cross-Talk Between ACh/nAChR and VEGF/VEGFR2 Systems In Vitro

To examine whether there is a potential relationship between the ACh/nAChR and VEGF/VEGFR2 systems, an analog of ACh (carbachol) and its receptor blockers were applied to HUVEC culture. As shown in Figure 6A and 6B, carbachol increased the levels of VEGF protein contained in the conditioned medium in a dose-dependent manner, whereas VEGFR2 phosphorylation followed a time-dependent manner. Profound suppression of carbachol-mediated VEGF secretion and VEGFR2 activation were observed in HUVECs treated with a nicotinic receptor blocker (methyllycaconitine; MLA). Pretreatment by atropine, in contrast, conferred no detectable change (Figure 6C and 6D). Furthermore, enhanced expression of HIF-1 $\alpha$  was detected in a time-dependent manner when HUVECs were exposed to carbachol (Figure 6E). Carbachol also stimulated p-Akt in a time-dependent manner, peaking at 30 minutes after administration (Figure 6F).



**Figure 4.** Donepezil-mediated eACh activity in vitro. After being cultured in serum-free EBM-2, HUVECs were treated with donepezil at a concentration of 10  $\mu\text{mol/L}$  for 24 hours. (A and B) Results of ChAT and VACHT gene expressions determined with real-time PCR. (C and D) Results of ChAT and VACHT protein expressions determined with Western blotting. Data of real-time PCR and Western blotting are represented as fold of control.  $n=4$  in each group. \* $P<0.05$ ; \*\* $P<0.01$ ; Student  $t$  test. (E and F) Representative images ( $\times 400$  magnification) of anti-ChAT and -VACHT immunofluorescence. Scale bar: 50  $\mu\text{m}$ . ChAT indicates choline acetyltransferase; eACh, endothelium-derived acetylcholine; EBM-2, endothelial basal medium 2; HUVECs, human umbilical vein endothelial cells; PCR, polymerase chain reaction; VACHT, vesicular acetylcholine transporter.



**Figure 5.** Donepezil-mediated angiogenic effect in vitro. (A) Quantitative analysis of HIF-1 $\alpha$  expression in lysates of HUVECs treated with donepezil (10  $\mu$ mol/L) and LY294002 (1 and 10  $\mu$ mol/L; Tukey's post-hoc test). (B) HIF-1 $\alpha$  expression in lysates of HUVECs when treated with nontargeting siRNA and siRNA HIF-1 $\alpha$  (100 nmol/L; Student *t* test). (C) ELISA for VEGF in the supernatant of HUVECs when treated with donepezil (10  $\mu$ mol/L) and HIF-1 $\alpha$  (100 nmol/L; Tukey's post-hoc test). (D and E) Quantitative analysis of stromal derived factor 1 (SDF-1) and basic fibroblastic growth factor (bFGF) contained in the supernatant of HUVECs (Student *t* test). *n*=4 in each group. Data of real-time PCR and Western blotting are represented as folds of control. \*\**P*<0.01. HIF-1 $\alpha$  indicates hypoxia-inducible factor 1 alpha; HUVECs, human umbilical vein endothelial cells; PCR, polymerase chain reaction; VEGFR, vascular endothelial growth factor receptor.

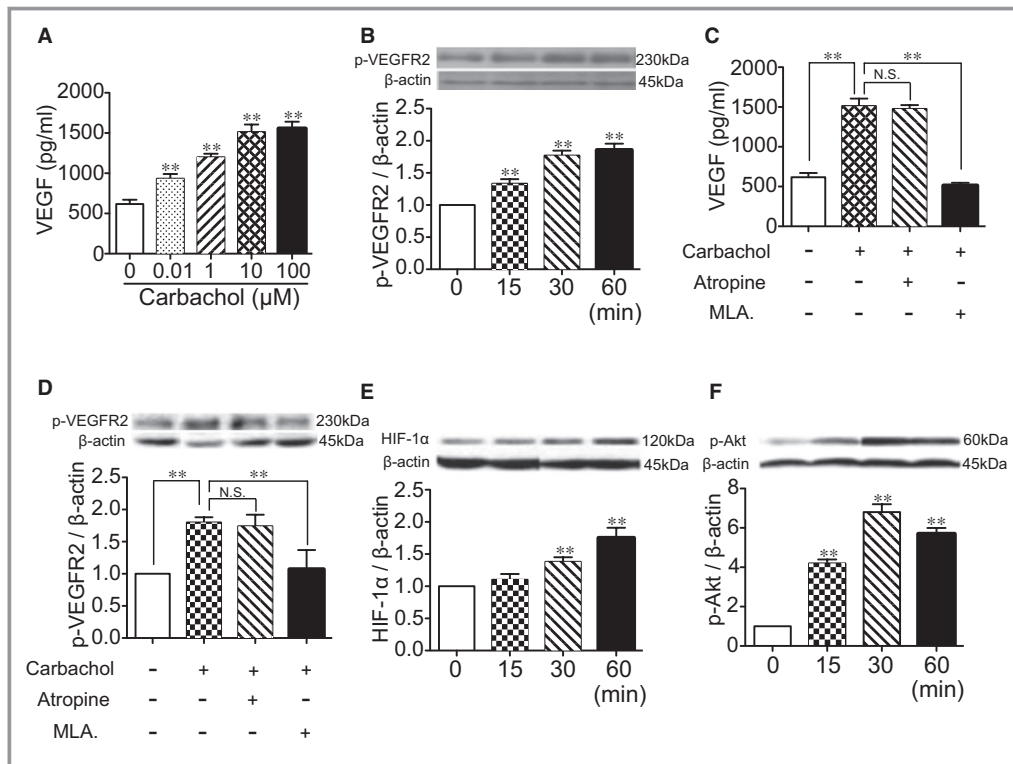
### Acetylcholinesterase Inhibition Accelerates Angiogenesis Even in $\alpha$ 7-nAChR KO with Hindlimb Ischemia

Previous reports using  $\alpha$ 7-nAChR KO mice indicated that  $\alpha$ 7-nAChR is highly involved in ischemia-induced angiogenesis. Therefore, to investigate whether the angiogenic effect of Pyr-mediated acetylcholinesterase inhibition is mediated through  $\alpha$ 7-nAChR, we studied the effects of Pyr on hindlimb ischemia using  $\alpha$ 7-nAChR KO mice. At first, we observed that  $\alpha$ 7-nAChR KO mice had a lower ischemic/normal limb temperature ratio and capillary formation in ischemic muscles than in wild-type (WT) mice (Figure 7A through 7D). Levels of VEGF were also lower in  $\alpha$ 7-nAChR KO mice than WT mice. Compared to untreated  $\alpha$ 7-nAChR KO mice, Pyr-treated  $\alpha$ 7-nAChR KO mice surprisingly exhibited an improved ischemic/normal limb temperature ratio and capillary density. Quantitative ELISA revealed that tissue VEGF levels were elevated in

Pyr-treated  $\alpha$ 7-nAChR KO mice than in untreated control mice (Figure 7E). In addition, Pyr augmented HIF-1 $\alpha$  expression in ischemic muscles (1.33-fold increasing versus untreated group).

### Discussion

In a previous study, impairment of ABR sensitivity led to sympathovagal imbalance and attenuated the adaption capacity of the cardiovascular system.<sup>10</sup> Moreover, dysfunctional ABR led to the acceleration of atherosclerosis in both apolipoprotein E KO and WT animals<sup>10,11</sup> and also decreasing the regulatory capacity of endothelial nitric oxide synthase.<sup>24,25</sup> In contrast, stimulation of the carotid sinus baroreflex prevents ventricular remodeling and improves endothelial function.<sup>26,27</sup> In our previous study, an enlarged infarcted area and declined ejection fraction were observed in the ischemic myocardium of SAD rats subjected to surgically



**Figure 6.** Effects of carbachol on HUVECs. HUVECs were exposed to different concentration gradients of carbachol (0 to 100  $\mu\text{mol/L}$ ) for 24 hours. (A) Concentrations of VEGF contained in the supernatant were measured by ELISA. After HUVECs were stimulated by carbachol (10  $\mu\text{mol/L}$ ), (B) levels of phosphorylation of VEGFR2 (p-VEGFR2; Tyr 996) were measured by Western blotting (\*\* $P < 0.01$  versus corresponding control; Tukey's post-hoc test). HUVECs were pretreated by atropine or methyllycaonitine (MLA) for 1 hour before carbachol (10  $\mu\text{mol/L}$ ) was added. Then, protein levels of (C) VEGF in supernatant and (D) p-VEGFR2 in cell lysates were quantified (\*\* $P < 0.01$ ; Tukey's post-hoc test). HUVECs were then exposed to carbachol (10  $\mu\text{mol/L}$ ) for 24 hours, and expression of (E) HIF-1 $\alpha$  and (F) phospho-Akt (p-Akt; Ser473) were measured by Western blotting (\*\* $P < 0.01$  versus corresponding control; Tukey's post-hoc test). Data of Western blotting are represented as folds of control.  $n = 4$  in each group. HIF-1 $\alpha$  indicates hypoxia-inducible factor 1 alpha; HUVECs, human umbilical vein endothelial cells; p-VEGFR2, phosphorylated vascular endothelial growth factor receptor 2.

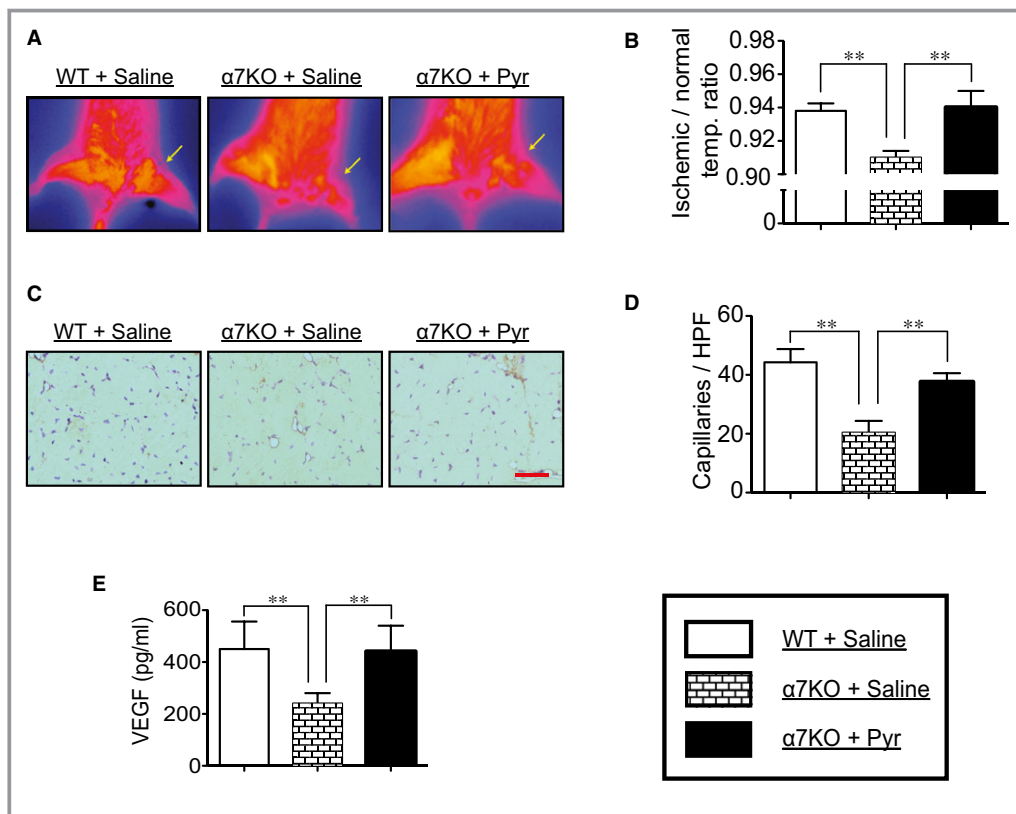
induced MI.<sup>12</sup> Clinically, decreased ABR sensitivity caused overaction of sympathetic tone and influenced the prognosis of CVDs.<sup>6–9</sup> In the present study of unilateral hindlimb ischemia, suppressed skin temperature ratio, capillary density, as well as reduced expressions of tissue VEGF and HIF-1 $\alpha$  were observed in the local area of SAD rats, indicating that dysfunctional ABR weakened endothelial function, thus delaying functional recovery and collateral vessel formation in response to ischemia (Figure 8).

On the other hand, ACh is no longer considered to be exclusively a neurotransmitter, simply mediating rapid communication between the neuron and effector cells, but is now seen as a hormone-like regulator that modulates various cell functions in such a way as to facilitate their adaption to new conditions.<sup>19–22,28</sup> Increasing the level of ACh by vagal stimulation protected ischemic cardiomyocytes from apoptosis through the Akt/HIF-1 $\alpha$  axis.<sup>29</sup> ACh also exhibited a

proangiogenic capacity that accelerated tube-like structure formation of HUVECs following a dose-dependent manner in vitro.<sup>12</sup> It has been clarified that in situ synthesis and secretion of ACh dominantly relies on the participation of ChAT and VACHT, respectively. Overexpression of either ChAT or VACHT raised the level of interstitial ACh.<sup>30,31</sup> In the present study, expressions of ChAT and VACHT declined in ischemic thigh of SAD rats, suggesting that dysfunctional ABR impairs the synthesis and secretion of endogenous ACh.

We hypothesized that restored sympathovagal balance by “drawing back” the ACh level would reactivate the angiogenic pathway and compensate for the impaired neovascularization caused by SAD intervention. We and other researchers have demonstrated that in experimental MI models, increasing parasympathetic tone by acetylcholinesterase inhibition augments postnatal neovascularization, preserves heart function, and increases survival.<sup>30,32</sup> In the present study, we applied





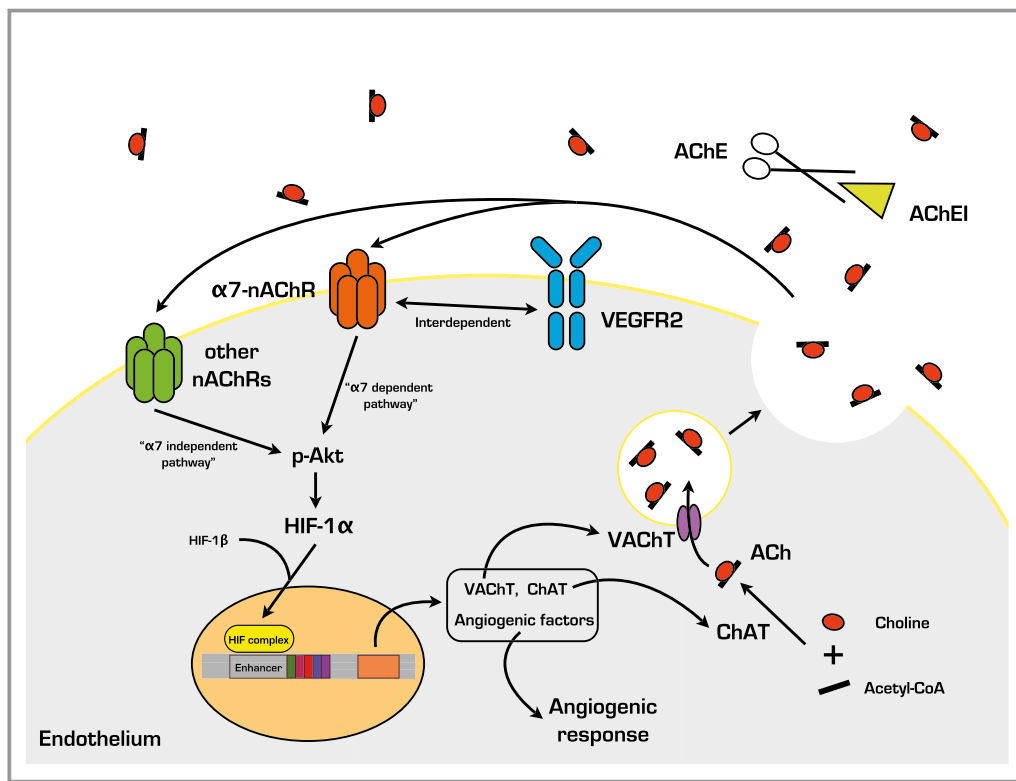
**Figure 7.** Pyridostigmine-mediated inhibition of acetylcholinesterase on angiogenesis. Using wild-type (WT) and  $\alpha 7$ -nAChR KO mice, unilateral hindlimb ischemia was surgically induced and fed by either pyridostigmine or saline to evaluate (A and B) the ischemic/normal limb temperature ratio, (C and D) the capillary density, and (E) levels of VEGF in ischemic muscles.  $n=6$  in each group.  $**P<0.01$ ; N.S., no significant difference; Tukey's post-hoc test.  $\alpha 7$ -nAChR indicates  $\alpha 7$ -nicotinic acetylcholine receptor; HPF, high power field; KO, knockout; Pyr, pyridostigmine; VEGFR, vascular endothelial growth factor receptor.

Pyr, an acetylcholinesterase inhibitor medicated for myasthenia gravis, to intensify the effect of ACh in ischemic tissue. Augmented angiogenic responses (skin temperature ratio and capillary formation), accompanied by restored local VEGF and HIF-1 $\alpha$  levels, were observed in the ischemic area of SAD rats after Pyr treatment. This indicates that acetylcholinesterase inhibition has persevered the limited ACh production under the circumstance of dysfunctional ABR status and, consequently, rebuilding blood perfusion by activating the HIF-1 $\alpha$ /VEGF axis in ischemic tissue.

It is becoming progressively more evident that the cholinergic system is not confined to the nerve, but is nearly ubiquitous.<sup>19–23</sup> Non-neural parts of the cholinergic system are highly involved in physiological homeostasis and in modulating cell functions. We and other researchers have reported that myocardium-derived ACh conferred resistance to ischemic heart disease and promoted survival.<sup>12,30</sup> Moreover, several reports have demonstrated the existence of an intact eACh system, including synthesis, secretion, and utilization.<sup>23,33–35</sup> The most significant observations of *in vivo* and *in vitro* studies were the enhancements of both gene and

protein expressions of ChAT and VAcHT under stimulation with an acetylcholinesterase inhibitor, suggesting the existence of positive feedback reinforcement in the production and utilization of eACh.

The receptor of ACh is also expressed on the surface of ECs, especially nAChR, the activation of which has been proven to exhibit proangiogenic potency.<sup>19,20,28</sup> We and other researchers have previously demonstrated that administration of a nicotinic receptor blocker could cancel ACh-mediated HUVEC vasculature formation *in vitro*; on the other hand, the muscarinic receptor blocker, atropine, caused only a modest effect.<sup>19</sup> Our observation here shows that administration of carbachol, an ACh analog, enhanced VEGF release from HUVECs and augmented expressions of p-VEGFR2 and HIF-1 $\alpha$  *in vitro*. Taken together, these results indicate that ACh not only triggers the paracrine function of ECs by activating the HIF-1 $\alpha$ /VEGF axis, but also promotes its autocrine function by activating VEGFR2 so that it magnifies the effect of VEGF. Moreover, we have shown that p-Akt was enhanced in response to ACh stimulation in a time-dependent manner *in vitro*, indicating that ACh triggers multiple pathways of ECs for



**Figure 8.** Possible mechanisms of acetylcholinesterase inhibitor-mediated angiogenesis in the present study. ACh indicates acetylcholine; AChEI, acetylcholinesterase inhibitor; ChAT, choline acetyltransferase; HIF-1 $\alpha$ , hypoxia-inducible factor 1 alpha;  $\alpha$ 7-nAChR,  $\alpha$ 7-nicotinic acetylcholine receptor; p-Akt, phosphor-Akt; VACHT, vesicular acetylcholine transporter; VEGFR, vascular endothelial growth factor receptor.

the adaptation of ischemic and hypoxic conditions. On the other hand, Heeschen et al. demonstrated exogenously that activation of nAChR by nicotine also triggers angiogenesis.<sup>18,20</sup> In an in vitro model of fibrovascular growth, nicotine-mediated angiogenic action was similar in magnitude to that of vascular growth factors, such as FGF.<sup>28</sup> In 2002, Heeschen et al. attempted to determine whether nAChR-mediated angiogenesis was mediated by VEGF or bFGF.<sup>20</sup> Neutralizing Abs against VEGF, but not bFGF, resulted in a significant, but not complete, inhibition of nAChR-mediated network formation. Further study showed that nAChR stimulation and selective stimulation of  $\alpha$ 7-nAChR both resulted in a significant release of VEGF.<sup>18</sup> Here, we have also demonstrated that in cultured HUVECs, nAChR stimulation by an ACh analog enhanced not only VEGF release, but also VEGFR-2 phosphorylation. Notably, antagonists of nAChR have been shown to markedly attenuate VEGF-induced angiogenesis.<sup>18</sup> Collectively, nAChR and VEGFR2 appear to mediate distinct, but interdependent, pathways of angiogenesis in response to ischemic stress.

We then administered donepezil to HUVECs to check the integration of eACh/nAChR with angiogenic signal pathways. Quantitative results demonstrated that donepezil treatment significantly raised the concentrations of several kinds of

angiogenic cytokines (VEGF, bFGF, and SDF-1) in the supernatant. Meanwhile, expression of HIF-1 $\alpha$  in HUVECs was augmented after treatment with donepezil. These data strongly suggest that the intact eACh/nAChR axis was conjugated with the angiogenic-signaling pathway of ECs. In addition, administration of donepezil augmented angiogenesis by stimulating release of insulin-like growth factor-1 in the hippocampus.<sup>36</sup> Consistent with results in vitro, Pyr increased protein levels of VEGF and HIF-1 $\alpha$  in rats with ABR dysfunction. Building on these findings, both ACh and its nicotinic receptor were present in ECs, and the eACh system was impaired by decreased ABR sensitivity. Therefore, pharmacological treatment by acetylcholinesterase inhibitor would maximize the effect eACh and enhance postnatal neovascularization.

$\alpha$ 7-nAChR expresses on the surface of ECs and is in charge of EC growth and migratory ability.<sup>19,28,37</sup> We previously reported that acetylcholinesterase inhibition promoted  $\alpha$ 7-nAChR expression in hypoxic myocardium through activation of the HIF-1 $\alpha$ /VEGF axis, which triggers angiogenesis.<sup>12</sup> In this study,  $\alpha$ 7-nAChR deletion impaired ischemia-induced angiogenic action. This change was then recovered by acetylcholinesterase inhibition with Pyr in a mouse ABR

dysfunction model, suggesting that acetylcholinesterase inhibitor-related vascular activity may not solely act through  $\alpha 7$ -nAChR in the lower extremities, but that other nAChRs are, at least partially, involved in the effect. It should be noted that there were no differences in  $\alpha 7$ -nAChR protein expression in ischemic muscles of the 3 groups of rats, whether treated or untreated with Pyr. It seems that Pyr-mediated vascular benefits in mice might not be attributable to quantitative change of  $\alpha 7$ -nAChR-dependent activation.

Besides  $\alpha 7$ -nAChR, there exists several other functional nAChRs expressed on ECs, and interplay between them modulates different cell functions.<sup>18,20,28</sup> Wu et al. described how nicotine remained capable of extending the tube length of HUVECs, even after  $\alpha 7$ -nAChR had been knocked down by siRNA in vitro. In the present study, we applied  $\alpha 7$ -nAChR KO mice to clarify the involvement of  $\alpha 7$ -nAChR in the mice model of PAD. Despite the comparison with WT mice,  $\alpha 7$ -nAChR KO exhibited decreased angiogenic response, and Pyr was still capable of triggering angiogenesis, even in the absence of  $\alpha 7$ -nAChR, in the mice model of hindlimb ischemia. Taken together, these results indicate that Pyr-mediated enhancement of ischemia-induced angiogenesis acts through both  $\alpha 7$ -nAChR-dependent and -independent pathways.

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## Disclosures

None.

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