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Acetylcholinesterase inhibitors attenuate atherogenesis in apolipoprotein E-knockout mice

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ABSTRACT

Objective: Donepezil, a reversible acetylcholinesterase inhibitor, improves cognitive function of Alzheimer's disease. Stimulation of cholinergic system was reported to improve long-term survival of rats with chronic heart failure and to attenuate inflammatory response in mice with lipopolysaccharide-induced sepsis. We sought to determine whether the pharmacological stimulation of cholinergic system by donepezil reduces atherogenesis in apolipoprotein (Apo) E-knockout (KO) mice.

Methods and results: Male ApoE-KO mice (10-week-old) were fed a high-fat diet and received infusion of angiotensin (Ang) II (490 ng/kg/day). Donepezil or physostigmine was administered for 4 weeks. Oral administration of donepezil (5 mg/kg/day) or infusion of physostigmine (2 mg/kg/day) significantly attenuated atherogenesis (Oil Red O-positive area) without significant changes in heart rate, blood pressure and total cholesterol levels. Administration of donepezil suppressed expression of monocyte chemoattractant protein-1 and tumor necrosis factor-α, NADPH oxidase activity and production of reactive oxygen species in the aorta.

Conclusion: The present study revealed novel anti-oxidative and anti-atherosclerotic effects of pharmacological stimulation of cholinergic system by donepezil. Donepezil may be used as a novel therapeutics for the atherosclerotic cardiovascular diseases.

1. Introduction

Activation of vagus nerve shows various effects on hemodynamics. It slows heart rate, dilates blood vessel and reduces blood pressure. Results of the Autonomic Tone and Reflexes After Myocardial Infarction Study and the Cardiac Insufficiency Bisoprolol Study II indicate that diminished cardiac vagus activity predicts the higher mortality rate in patients with chronic heart failure [1,2]. In addition, vagus nerve stimulation (VNS) improves long-term survival of rats with chronic heart failure after experimental myocardial infarction [3]. VNS modulates the cardiac redox status and adrenergic drive, and thereby suppresses free radical generation in the failing heart [4]. However, the effect of VNS on vascular lesion formation has not been reported.

Stimulation of cholinergic system was reported to attenuate tumor necrosis factor (TNF)-α production from macrophages and hypotensive shock in a lipopolysaccharide (LPS)-induced septic model [5,6]. Stimulation of cholinergic system inhibits activation of nuclear factor-kappa B (NF-κB) [7] and induces suppressor of cytokine signal 3 expression [8], resulting in the attenuation of inflammatory responses. However, nicotine, a nicotinic acetylcholine receptor (AchR) agonist, was reported to induce endothelial dysfunction that is an initial step of atherosclerosis and to accelerate atherosclerosis in Apolipoprotein E-knockout (ApoE-KO) mice [9]. Therefore, it is not clear whether the activation of cholinergic system is atherogenic or atheroprotective.

Donepezil [diethyl(3,5-di-ter-butyl-4-hydroxybenzyl)phosphonate] is a long acting, reversible cholinesterase inhibitor and is known to improve memory and cognitive function in patients with Alzheimer's disease [10]. A recent study showed that treatment of patients with Alzheimer's disease with donepezil for 1 month reduces production of oncostatin-M, interleukin (IL)-6 and IL-1 in the peripheral blood mononuclear cells [11], suggesting a possible anti-inflammatory effect of donepezil. However, the mechanism remains to be determined.

Angiotensin (Ang) II plays critical roles in the progression of atherosclerosis, ventricular remodeling after myocardial infarction and heart failure [12]. One of the mechanisms by which AngII

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accelerates atherogenesis is the induction of oxidative stress and inflammation [13]. AngII activates NADPH oxidase in the blood vessel resulting in the activation of redox-sensitive transcription factors such as nuclear factor (NF)-B and activating protein (AP)-1 [14], resulting in the production of inflammatory cytokines or chemokines such as TNF-α, IL-6, and monocyte chemoattractant protein (MCP)-1.

These previous studies prompted us to examine the effect of pharmacological stimulation of cholinergic system by donepezil on the progression of atherosclerosis in ApoE-KO mice. In the present study we showed that donepezil attenuated atherogenesis in ApoE-KO mice fed a high-fat diet (HFD) with or without AngII stimulation, possibly through anti-oxidative and anti-inflammatory effects.

2. Materials and methods

2.1. Materials

AngII was purchased from PEPTIDE Institute Inc. Physostigmine, Ach, lucigenin, β-nicotinamide adenine dinucleotide 2′-phosphate reduced hydrate (NADPH) were purchased from Sigma Chemical Co. Donepezil was purchased from Toronto Research Chemicals Inc. Antibodies against p47phox and NOX1 were purchased from Santa Cruz Biotechnology, Inc. Other chemical reagents were purchased from Wako Pure Chemicals, unless mentioned specifically.

2.2. Animal model of atherosclerosis

All procedures were approved by the committee on Ethics of Animal Experiment, Kyushu University Graduate School of Medical Sciences and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

C57BL/6J ApoE-KO mice were purchased from the Jackson Laboratory. Male ApoE-KO (10-week-old) mice were fed a HFD (35% calorie from fat, 1% cholesterol) and received infusion of AngII (490 ng/kg/day) through an osmotic minipump (Alzet) implanted in the peritoneal cavity for 4 weeks. Mice had an ad libitum access to both food and water. Four groups were compared: control, AngII + HFD, AngII + HFD and donepezil (estimated dose of ingestion: 5 mg/kg/day via drinking water), and AngII + HFD and physostigmine (2 mg/kg/day via second osmotic minipump). Blood pressure and heart rate were monitored using a computed tail-cuff system (UR-5000, UEDA, Ueda Co. Ltd.). The doses of cholinesterase inhibitors were chosen on the basis of previous studies that showed that donepezil [15] or physostigmine [16] at the doses mentioned above did not affect heart rate or blood pressure level in mice.

In another experiment, ApoE-KO mice (12-week-old) were fed a HFD only for 8 weeks without AngII. And the effect of donepezil was examined.

2.3. Histological and immunohistochemical analyses

At the end of experiments, mice were anesthetized with an intraperitoneal injection of pentobarbital. The circulatory system was perfused with PBS via the left ventricle. Then, the aortic arch and the thoracic aorta was opened longitudinally, stained with Oil Red O, and pinned out on a black wax surface. The percentage of the plaque area stained by Oil Red O to the total luminal surface area was determined. Serial sections of the aortic root were prepared and were stained with the antibodies against macrophage (F4/80; Serotec Inc.) and MCP-1 (Santa Cruz Biotechnology Inc.). All images were captured with a Nikon microscope equipped with a video camera and analyzed using Adobe Photoshop and Scion Image Software.

2.4. Tissue preparation

The thoracic and abdominal aorta were immediately frozen in liquid nitrogen for RNA isolation, Lucigenin assay, and Western blot analysis. For RNA isolation, thoracic aorta was additionally perfused with RNA Later (Ambion) to prevent RNA degradation. Frozen samples of thoracic aorta were crashed on dry ice and homogenized in ISOGEN (Nippon Gene) and total RNA was prepared in accordance with the manufacturer's instruction.

2.5. Real-time reverse transcription polymerase chain reaction analysis

Reverse transcription of RNA was performed with ReverTra Ace (TOYOBO). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using SYBR Green and the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The sequences of PCR primers used in this study are summarized in Supplemental Table 1. Primers for GAPDH were purchased from ABI, of which sequences are not disclosed.

2.6. Lucigenin-enhanced chemiluminescence assay

NADPH-dependent superoxide production was measured by lucigenin luminescence [17]. The aorta was perfused with ice cold PBS, immediately frozen in liquid nitrogen and the assay was performed on the same day. The frozen samples of abdominal aorta were crashed on dry ice and homogenized in modified Krebs buffer (99 mmol/L NaCl, 4.7 mmol/L KCl, 1.9 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.0 mmol/L K_2HPO_4, 25 mmol/L $NaHCO_3$, 20 mmol/L Na-HEPES, 11 mmol/L D-glucose). A luminescence assay was performed in a balanced salt solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HpO_4, 1.5 mmol/L KH_2PO_4) buffer containing 5 μmol/L of lucigenin using a luminescence reader (Berthold Technology). The reaction was started by adding 100 μmol/L of β-NADPH as a substrate.

2.7. Oxidative fluorescent microphotography

Superoxide was detected in the layers of the vessel wall using fluorescent probe dihydroethidium (DHE; Molecular Probes) as described previously [18]. After perfusion with ice cold PBS, the ascending thoracic aorta was immediately frozen in OCT compound (Sakura Finetek) and stored at −80 °C until preparation for the cryosection. Cryosections (10 μm) were prepared in the next day and incubated for 30 min at 37 °C with 2 μmol/L DHE. Images were obtained on a confocal microscope (excitation filter at 488 nm; emission filter at 550 nm).

2.8. Western blot analysis

The aorta was homogenized in modified Krebs buffer. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co). The homogenates were heated in a sample buffer at 95 °C for 3 min, electrophoresed on 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). Western blot analysis of p47phox, NOX1 and α-tubulin was performed by a conventional method and detected by ECL chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Membranes were scanned using LAS-4000mini bioimage analyzer (Fujifilm).