Vascular Pharmacology 61 (2014) 49–55

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Stimulation of α7 nicotinic acetylcholine receptor by AR-R17779 suppresses atherosclerosis and aortic aneurysm formation in apolipoprotein E-deficient mice

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ARTICLE INFO

Article history:
Received 5 February 2014
Received in revised form 10 March 2014
Accepted 20 March 2014
Available online 29 March 2014

Keywords:
Atherosclerosis
Inflammation
Cholinergic anti-inflammatory pathway
α7-Nicotinic acetylcholine receptor
Angiotensin receptor

ABSTRACT

Atherosclerosis is a chronic inflammatory disease. It has been appreciated that vagus nerve inhibits macrophage activation via α7 nicotinic acetylcholine receptor (nAChR), termed the cholinergic anti-inflammatory pathway. We explored the effects of AR-R17779, a selective α7nAChR agonist, on atherosclerosis and aneurysm formation in apolipoprotein E (ApoE)-deficient mice. ApoE-deficient mice were fed a high-fat diet (HFD) and angiotensin II (Ang II) was infused by osmotic minipumps from 10-week-old for 4 weeks. AR-R17779 was given in drinking water ad libitum. Oil red O staining of the aorta showed that combined loading of HFD and Ang II induced marked atherosclerosis compared with control mice fed a normal chow. Treatment with AR-R17779 significantly reduced atherosclerotic plaque area and improved survival of mice. Treatment with AR-R17779 also suppressed abdominal aortic aneurysm formation. Quantitative RT-PCR of the aorta revealed that mRNA expression levels of interleukin-1β, interleukin-6 and NOX2 were significantly decreased in AR-R17779-treated mice compared with Ang II + HFD mice. AR-R17779 treatment also reduced blood pressure and serum lipid levels. In conclusion, α7nAChR activation attenuates atherogenesis and aortic abdominal aneurysm formation in ApoE-deficient mice possibly through an anti-inflammatory effect and reduction of blood pressure and lipid levels. Pharmacological activation of α7nAChR may have a therapeutic potential against atherosclerotic vascular diseases through multiple mechanisms.

1. Introduction

Atherosclerosis progresses insidiously, and often results in life-threatening catastrophe such as sudden cardiac arrest, myocardial infarction, and stroke. Although there are effective modalities for the primary and secondary prevention of atherosclerotic cardiovascular diseases, cardiovascular events remain major causes of death in the developed countries [1]. In addition to adequate management of cardiovascular risk factors, therapeutic strategies based on the pathophysiology of atherosclerosis might improve the prognosis of patients with cardiovascular diseases. It has been appreciated that atherosclerosis is a chronic inflammatory disease [2], to which both innate immunity and adaptive immunity contribute [3]. Inflammatory cells including monocytes/macrophages, mast cells, dendritic cells, and T lymphocytes are involved in both initiation and progression of atherosclerosis, which is mediated by proinflammatory cytokines, chemokines, growth factors, and adhesion molecules [2–4].

Inflammation is a physiological process equipped to protect organisms from pathogenic threats. Excessive inflammatory responses, however, could be more harmful than the original stimuli. Exaggerated inflammation causes morbidity and mortality in diseases including endotoxin shock, rheumatoid arthritis, inflammatory bowel disease, diabetes, atherosclerosis, myocardial and cerebral ischemia [5]. It has been well known that endogenous anti-inflammatory humoral mechanisms prevent excessive inflammatory responses and maintain homeostasis [6]. Recently, Borovikova et al. reported that stimulation of the vagus nerve attenuates macrophage activation and systemic inflammatory response [7]. Thereafter, many studies have demonstrated that electrical stimulation of the vagus nerve attenuates inflammatory diseases including arthritis, ischemia/reperfusion injury, hemorrhagic shock, and postoperative ileus in animal models [8–13]. In contrast, vagotomy exacerbates arthritis, endotoxemia, hemorrhagic shock, septic peritonitis, inflammatory bowel disease, and pancreatitis [8,

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http://dx.doi.org/10.1016/j.vph.2014.03.006
1537-1891/

14–18]. This vagally-mediated anti-inflammatory mechanism has been designated "cholinergic anti-inflammatory pathway" [5]. α7 nicotinic acetylcholine receptor (α7nAChR) on immune cells is a key mediator of the cholinergic anti-inflammatory pathway [19]. Acetylcholine released from efferent vagus nerve ending stimulates α7nAChR, which attenuates inflammatory responses through activation of the JAK2–STAT3 pathway and suppression of the NF-κB pathway [13,20].

In the present study, we explored the involvement of α7nAChR pathway in atherogenesis and examined the effects of a selective α7nAChR agonist, AR-R17779, on an angiotensin II (Ang II)-accelerated atherosclerosis model in apolipoprotein E (ApoE)-deficient mice. We found that treatment with AR-R17779 improved survival of mice, reduced atherosclerotic plaque formation and suppressed development of abdominal aortic aneurysm in ApoE-deficient mice.

2. Materials and methods

2.1. Materials

Ang II was purchased from Peptide Institute, Inc. (Osaka, Japan). AR-R17779 was a gift from CVGI iMED, AstraZeneca R&D Mölndal, Sweden. Other chemical reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated.

2.2. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by the acid guanidinium thiocyanate–phenol chloroform extraction method. RNA was reverse-transcribed using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression levels were determined by comparative Ct (ΔΔCt) method. Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) mRNA was used for standardization. Primer sequences used for amplification are as follows: interleukin(*IL*)-*6* (forward) 5′ CCACTTCA CAAGTCGGAGGCTTA 3′, (reverse) 5′-GCAAGTGCATCATCGTTGTTCA TAC-3′; *IL-1β* (forward) 5′-TCCAGGATGAGGACATGAGCAC-3′, (reverse) 5′-GAACGTCACACACCAGCAGGTTA-3′; tumor necrosis factor (*Tnf*)*α* (forward) 5′-AAGCCTGTAGCCCACGTCGTA-3′, (reverse) 5′-GGCACCAC TAGTTGGTTGTCTTTG-3′; *Nox2* (forward) 5′-CCAACTGGGATAACGAGT TCAAGAC-3′, (reverse) 5′-AAGGCTTCAGGGCCACACA 3′; angiotensin II type 1a receptor (*AT1aR*) (forward) 5′-GGACACTGCCATGCCCATAAC-3′, (reverse) 5′-TGAGTGCGACTTGGCCTTTG-3′; and *Hprt1* (forward) 5′-TTGTTGTTGGATATGCCCTTGACTA-3′, (reverse) 5′-AGGCAGATGGCC ACAGGACTA-3′.

2.3. Animal experiments

All procedures were approved by the Institutional Animal Use and Care Committee, and conducted in accordance with Institutional Guidelines and Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Male ApoE-deficient mice (B6.129P2-*ApoE*^{tm1Unc}/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). At 10-week-old, mice were anesthetized by a bolus intraperitoneal injection of 90 mg/kg ketamine and 4 mg/kg of xylazine. Then the mice had mini-osmotic pumps (Alzet, DURECT, Cupertino, CA, USA) delivering Ang II (1.9 mg/kg/day) implanted into the intraperitoneal space. The mice were fed a high-fat diet (HFD, 1% cholesterol, 35% calorie from fat) since. AR-R17779 was administered in drinking water ad libitum, yielding a daily estimated dose of 20 mg/kg. Body weight, blood pressure and heart rate were measured at day 0 and day 28. Blood pressure and heart rate were monitored non-invasively by a tail cuff method (UR-5000, UEDA, Ueda Co. Ltd. Tokyo, Japan). After 4 weeks of treatment, mice were euthanized humanely by overdose injection of pentobarbital. Harvested tissues were fixed in 10% neutral-buffered formaldehyde solution or snap-frozen in liquid nitrogen for RNA isolation.

2.4. Morphometric analysis and immunohistochemistry

The circulatory system was perfused with PBS via the left ventricle. Then, the aortic arch and the thoracic aorta were 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 by NIH ImageJ software. Maximum diameter of the abdominal aorta was measured and then total RNA was extracted from supra renal portion of the abdominal aorta.

2.5. Measurement of serum cholesterol and triglyceride levels

Serum triglyceride and total cholesterol levels were determined by Triglyceride E-test Wako (Wako) and Cholesterol E-test Wako (Wako), respectively.

2.6. Measurement of serum AR-R17779 level

Serum samples were precipitated with acetonitrile. After centrifugation, the supernatant was diluted 1:1 with water. Analysis was performed on a short reversed-phase C18 high performance liquid chromatography column (Xbridge C18 3.0 cm × 2.1 mm, 2.5 μm, Waters Corporation, MA, USA) with rapid gradient elution, and tandem mass spectrometry detection using a triple quadrupole instrument with electrospray ionization and multiple reaction monitoring acquisition.

2.7. Cell culture

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of Sprague–Dawley rat by an explant method and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air–5% CO_2. VSMCs were grown to confluence, cultured in DMEM with 0.1% BSA for additional 2 days and used in the experiment. Cells between passages 4 and 10 were used.

2.8. Statistical analysis

Experimental data were analyzed by one-way ANOVA and Fisher's *post hoc* test. Results are expressed as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. Differences in survival rates among groups were evaluated by log-rank test.

3. Results

3.1. Treatment with AR-R17779 attenuates atherogenesis in ApoE-deficient mice

Combined loading of a HFD and Ang II infusion induced hypertension and severe hypercholesterolemia, and developed marked atherosclerotic lesions in the aorta of ApoE-deficient mice (Table 1 and Fig. 1). Treatment with AR-R17779 significantly reduced atherosclerotic plaque area in the thoracic aorta (control: 3.5 ± 0.6%, Ang II + HFD: 28.6 ± 0.5%, Ang II + HFD + AR 18.4 ± 1.4%, $p < 0.001$) (Fig. 1A and B). AR-R17779 also lowered blood pressure, heart rate, serum total cholesterol level, and serum triglyceride level compared with Ang II + HFD mice (Table 1). No sickness behavior or apparent abnormalities were observed during the experimental period in AR-R17779-treated mice. The serum concentration of AR-R17779 at the end of experiment was 1.18 ± 0.17 μM. Unexpectedly, quantitative analysis of macrophage infiltration by immunohistochemical staining for Mac-3 failed to show a decrease in macrophage accumulation (Fig. 1C and D).