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Pseudolaric acid B attenuates atherosclerosis progression and inflammation by suppressing PPARγ-mediated NF-κB activation

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ABSTRACT

Aims/objective: Atherosclerosis is a progressive disease of large arteries characterized with chronic inflammation and aberrant immune response. Pseudolaric acid B (PB) has been found to exert multiple effects by inhibiting inflammatory response. However, there is no comprehensive assessment of the effects of PB on atherosclerosis using relevant *in vivo* and *in vitro* models.

Material and methods: Male ApoE$^{-/-}$ mice were treated with PB orally with a high fat diet (HFD) to clarify its anti-atherosclerotic activities. RAW264.7 macrophage line, a well-accepted cell model of atherosclerosis, was used to investigate anti-inflammatory effects and molecular mechanisms of PB.

Results: PB significantly attenuated atherosclerotic lesions by modulating plasma lipid profiles as well as inhibiting inflammatory responses in macrophages of atherosclerotic mice. Meanwhile, PB markedly suppressed the expression of pro-inflammatory cytokines, and regulated cholesterol efflux related genes in oxidative low density lipoprotein (ox-LDL)-loaded macrophages. The cellular uptake of Dil-labeled ox-LDL was significantly inhibited by PB either. Moreover, the ability of PB to suppress nuclear factor kappa B (NF-κB) and activate peroxisome proliferator-activated receptor gamma (PPARγ) was confirmed using luciferase reporter assays. Conversely, the selective PPARγ antagonist GW9662 reversed the influence of PB in macrophages.

Conclusion: Together, these findings indicate that PB exerts its protective effects on atherosclerosis by inhibiting macrophage-mediated inflammatory response and cellular ox-LDL uptake, and promoting cholesterol efflux by suppressing NF-κB activation PPARγ-dependently. Therefore, PB may be a promising agent for inflammatory and atherosclerotic diseases.

1. Introduction

Atherosclerosis is a leading cause of cardiovascular disease and stroke, which is characterized with chronic inflammation and aberrant immune response involved with lipid metabolism. Thus, therapeutic modulation to regulate inflammation and the immune system response may prove to be very promising strategies in the management of atherosclerosis [1]. A considerable portion of new drugs are from plant-derived natural products and their synthetic mimics, among which terpenoids are the largest known class of secondary metabolites in plants and possess a broad range of biological activities including potential therapeutic effects [2]. Pseudolaric acid B (PB) is a diterpene acid isolated from the extract of the root bark of *Pseudolarix kaempferi Gordon* (pinaceae), which includes the molecular structure of a compact tricyclic core containing a fused ring system (polyhydroazulene) and 4 contiguous stereocenters. The structural features suggest that PB may have important pharmacological effects on inflammation [3,4]. However, little is known about its underlying molecular mechanisms involved in inflammatory response, and the roles of PB on atherosclerosis have not been elucidated.

Abbreviations: ABCA, ATP-binding cassette transporter A; Dil-oxLDL, 1,1′-dioctadecyl-3,3,3′3′-tetra-methylindocyanide percholorate-labeled ox-LDL; HDL-C, high-density lipoprotein-cholesterol; IL, interleukin; LDL-C, low-density lipoprotein-cholesterol; LXR, Liver X Receptor; NF-κB, Nuclear factor kappa B; ox-LDL, Oxidized low-density lipoprotein; PPARγ, peroxisome proliferator-activated receptor gamma; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor α

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Atherosclerosis-susceptible apolipoprotein E–deficient ($ApoE^{-/-}$) mouse model is a widely recognized model, displaying poor lipoprotein clearance with subsequent accumulation of cholesterol ester-enriched particles in the blood with properties similar to those observed in human [5]. Especially, $ApoE^{-/-}$ mice fed with high-fat diet (HFD) could show markedly accelerated plaque development [6]. Thus, the present study was designed to investigate the protective effects of PB on atherosclerotic plaque development in $ApoE^{-/-}$ mice kept on a HFD. Moreover, the underlying molecular mechanisms involved in the inflammatory response of PB were conducted in ox-LDL-loaded macrophages. These findings might shed more light on the potential of natural products-based treatment strategies for atherosclerosis.

2. Materials and methods

2.1. Materials

PB was provided by professor Chen (Logistics University of the Chinese People's Armed Police Force), which purity was > 98% determined by HPLC analysis [7]. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). 2-chloro-5-nitro-*N*-phenyl-benzamide (GW9662, ≥98% pure), Oil Red O, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thioglycollate was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Anti-mouse Ly6G-peridinin chlorophyll protein-Cy5.5 (PerCP/Cy5.5), CD11b-phycoerythrin (PE), and Ly-6C-fluorescein isothiocyanate (FITC) antibodies were purchased from Biolegend (San-Diego, CA, USA). Anti-mouse LXRα, ABCA1, PPARγ, CD36, F4/80, Ki67, GAPDH, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Abcam (Cambridge, UK). Enhanced Chemiluminescence (ECL) kit and Bicinchoninic Acid (BCA) protein assay kit were purchased from Pierce Biotechnology. Mouse interleukin (IL)-1β and TNF-α Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Dakewe Biotech (Shen-zhen, China). Oxidized low-density lipoprotein (ox-LDL) and 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocyanide percholorate (Dil)-labeled ox-LDL (Dil-oxLDL) were obtained from Biomedical Technologies (MontgomeryVillage, MD, USA).

2.2. Animals and experimental protocol

Male C57/BL6 mice and $ApoE^{-/-}$ mice (C57/BL6 genetic background), 8 weeks of age, were purchased from Vital River Laboratories Animal Center (Beijing, China). Mice were maintained on a dark/light cycle (12/12 h) in air-conditioned rooms (22.5 ± 0.5 °C, 50 ± 5% humidity) and were adapted to local conditions for one week before the beginning of this study. All animal experiments were conducted according to national and international laws and policies and were approved by the Institutional Animal Care and Use Committee of Logistics University of the Chinese People's Armed Police Force.

Male $ApoE^{-/-}$ mice were fed with HFD containing 21.2% fat and 0.2% (w/w) cholesterol obtained from TROPHIC Company (Jiangsu, China; Diet Number TP26303) for a duration of 8 weeks, and were randomly divided into two groups with a mean initial body weight (n = 12 mice/group): PB was administered intragastrically at a dose of 5 mg/kg once a day continuously for 4 weeks, while the control group received an equal volume of normal saline (NS).

2.3. Determination of lipid profile

The mice were anesthetized and euthanized, then plasma was separated by centrifugation at 3000 rpm for 15 min at 4 °C. Total cholesterol (TC) and triglyceride (TG) were measured by commercially available ELISA kits (Biolab Technology Co., Ltd., BeiJing, China). The absorbance at 450 nm was read using a microplate reader (Bio-Rad Model 680). The levels of plasma low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) were determined by enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

2.4. Analysis of atherosclerotic lesions

The whole aortas from the thoracic to the abdominal aortas of mice were dissected, and the part of the hearts just below the atria including the aortic root and the base of the hearts were embedded in OCT compound, snap frozen and stored at −80 °C. After trimming, successive 5-μm transversal sections of the aortic sinus were obtained using a cryotome (CM7500, Leica, Germany). Lipid accumulation was observed by Oil Red O staining. The surface area of the atherosclerotic plaques relative to the total aortic surface area was quantified by histomorphometric analysis. All images were captured with an Olympus BX41 microscope equipped with a video camera and analyzed using Image–Pro-Plus software (version 6.0, Media Cybernetics, MD, USA). Two observers performed the measurements independently and blindly.

2.5. ELISA for inflammatory cytokines

The isolated aortic tissues were homogenized in ice-cold lysis buffer containing protein inhibitor. Concentrations of TNF-α and IL-1β in the serum and homogenized samples were quantified by ELISA assay. The data were shown as picograms per millilitre (pg/ml).

2.6. Flow cytometric analysis

Red blood cells of mice were lysed with erythrocyte lysis buffer (0.15 M NH4Cl, 10 mM NaHCO3 and 0.1 mM EDTA; pH 7.3). Subsequently, 0.3 million cells per sample were stained with the appropriate antibodies to discriminate monocytes: Ly6G-PerCP/Cy5.5, CD11b-PE and Ly6C-FITC. Monocyte subsets were further delineated by fluorescent-activated cell sorter (FACS) analysis using Ly6C into inflammatory ($Ly6C^{hi}$) and $Ly6C^{lo}$, and the gating strategies were performed as previously described with some modifications [8]. Data were acquired on a FACScan flow cytometer (CytomicsTM FC 500, Beckman Coulter, USA) and subsequently analyzed with FlowJo7 software (Treestar, Ashland, OR, USA).

2.7. Immunofluorescence

Cryostat 5-μm cross-sections of the aortic valves were incubated with anti-mouse F4/80 (1:100) and Ki67 (1:300) for overnight incubation at 4 °C in a humidity chamber, and then incubated with goat anti-rat or goat anti-rabbit secondary antibody (1:100) for 1 h at room temperature. Following washing, nuclear staining with DAPI was carried out for 15 min. Fluorescence imaging of mice macrophages was analyzed by a confocal laser-scanning microscope (Leica TCS SP8, Germany). Settings for image acquisition were identical for control and experimental tissues. Data were expressed as the percent of macrophage proliferation using Image-Pro Plus 6.0 software.

2.8. Cell culture and treatment

RAW264.7 cells (murine macrophage cell line) were obtained from the American Type Culture Collection (ATCC, TIB-71) and maintained in DMEM medium containing glucose at the concentration of 4500 mg/l and supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). After overnight incubation at 37 °C in 5% CO_2, the culture medium was removed, and the adherent cells were washed twice with sterile phosphate-buffered saline (PBS, Thermo Scientific) before experimental manipulations. Primary mouse peritoneal macrophages were elicited from C57BL/6 mice by intraperitoneal injection of