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Research Paper

Anti-atherosclerotic effects of *Polygonum aviculare* L. ethanol extract in ApoE knock-out mice fed a Western diet mediated via the MAPK pathway

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ABSTRACT

Ethnopharmacological relevance: *Polygonum aviculare* L. has been used in traditional Korean medicine to treat obesity and symptoms associated with hypertension. The effectiveness or mechanism of *Polygonum aviculare* L. ethanol extract (PAE) on atherosclerosis disease has not been examined experimentally. This study investigated the protective effect of PAE in atherosclerotic mice.

Materials and methods: ApoE KO mice were fed a Western diet (WD) alone or with PAE or a statin for 12 weeks, followed by analysis of bodyweight, serum lipid levels, and blood pressure. Staining of the aorta and adipose tissue, expression levels of adhesion molecules, and the MAPK pathway were also examined. Cell viability, NF-κB activity, and protein levels of adhesion molecules were assessed in vitro.

Results: ApoE KO mice fed PAE (50 and 100 mg/kg) or statin (10 mg/kg) gained less body weight, and has less adipose tissue and lower serum lipid levels and blood pressures than the WD group. Aorta ICAM-1, VCAM-1, and NF-κB levels were decreased by PAE in a dose-dependent manner, consistent with the in vitro observations. PAE and statin decreased atherosclerotic plaque and adipocyte size versus the WD group. Furthermore, PAE decreased phosphorylation of MAPK pathway components in the aorta of PAE-treated mice, suggesting that PAE's anti-atherosclerotic effects are mediated via a MAPK pathway-dependent mechanism.

Conclusions: PAE may protect against the development of atherosclerotic disease. The beneficial effects are associated with lowering bodyweight, serum lipids, blood pressure, adhesion molecular protein levels, atherosclerotic plaque, and adipocyte size, involving the MAPK pathway.

1. Introduction

Atherosclerosis, a chronic inflammatory disease affecting the large and medium-size arteries, is associated with vascular dysfunction in obesity and increased rates of cardiovascular death. There are many plausible mechanisms by which an increase in adipose tissue could adversely affect vessel walls. These include changes in blood pressure, glucose levels, lipid/lipoprotein metabolism, and systemic inflammation (Fantuzzi and Mazzone, 2007). Also, in terms of it being a chronic inflammatory disease, this results from the increased production of adhesion molecules and various cytokines, such as tumor necrosis factor-α (TNF-α), and transcription factor nuclear factor-κB (NF-κB) (Getz and Reardon., 2012; Kim et al., 2013). Therapies that target vascular inflammation are efficacious in atherosclerosis, suggesting that vascular inflammation is an important determinant of atherosclerosis outcome.

In traditional Korean herbal medicine, *Polygonum aviculare* L. (Polygonaceae family) has been used as an anti-hypertensive and anti-obesity agent in treating cardiovascular disease (Yin et al., 2005; Sung et al., 2013). Several reports have described beneficial vasorelaxant effects of PAE on pre-contracted rat aortic tissues (Yin et al., 2005), scavenging activity for free radicals and superoxide, and inhibition of lipid peroxidation in mouse brain induced by ferric ions plus ascorbic acid (Hau, 2006). The *Polygonum aviculare* L. plant has also been used in traditional medicine for the treatment of hemorrhage and hemoptysis, and as a coagulant and sedative treatment for many symptoms associated with hypertension (Habibi et al., 2001; Yin et al., 2005). Additionally, the plant has been prescribed traditionally to treat inflammatory diseases, such as arthritis and bronchitis (Howard, 1987).

Abbreviations: HASMC, human aortic smooth muscle cell; TNF-α, tumor necrosis factor-α; ApoE, apolipoprotein E; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; NF-κB, nuclear factor-κB; KIOM, Korea Institute of Oriental Medicine

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However, no previous report has described protective effects of PAE against vascular inflammation associated with atherosclerosis in a Western diet-fed ApoE KO mouse model.

In the present study, we examined the protective effects of PAE extracts on vascular inflammation in Western diet (WD)-fed ApoE KO mice and further explored the possible protective mechanism of PAE in atherosclerosis. We investigated the effects of PAE on body and adipose tissue weight, lipid levels, blood pressure, and inflammatory signaling in aorta tissue using a mouse atherosclerosis model. The findings suggest that PAE has protective effects against aorta damage in atherosclerosis through an anti-vascular inflammation mechanism. Our results provide a scientific basis supporting the traditional medical use of PAE in atherosclerosis.

2. Materials and methods

2.1. Preparation of Polygonum aviculare L. extract

Polygonum aviculare L. was purchased from Omniherb Co. (Yeongcheon, Gyeongsangbuk-do, Republic of Korea) and was authenticated based on its microscopic and macroscopic characteristics by the Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM), Daejeon, Republic of Korea. A voucher specimen (no. JA-38) was deposited at the herbarium of the Herbal Material Management group at KIOM. Dried *Polygonum aviculare* L. (100 g) was extracted twice with 70% ethanol (with a 2-h reflux). The extract was concentrated under reduced pressure at 40 °C using a rotary evaporator. The decoction was filtered, lyophilized, and stored at 4 °C until use (Sung et al., 2013). The yield of the dried extract from the starting crude materials was approximately 14.03% (w/w). The solid form of the extract was dissolved in dimethyl sulfoxide (DMSO).

2.2. Cell culture

HASMCs were purchased from ScienCell Research Laboratory (San Diego, CA, USA). The cells were cultured as monolayers in smooth muscle cell medium (ScienCell) containing essential and nonessential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals, and 2% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Cells from passages 2 to 6 were used in this study.

2.3. Cell viability

The MTT assay is a standard method to assess cell viability. HASMCs (1×10^5) were seeded in 96-well microtiter plates. The cells were treated with various concentrations of *PAE* (50, 100, 250 μg/mL) for 24 h. Subsequently, 100 μL MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated at 37 °C for 4 h, and 200 μL DMSO was added to dissolve the formazan crystals. The absorbance was measured at 540 nm using a spectrophotometer.

2.4. NF-κB activity assay

NF-κB activity was measured by luciferase reporter assays. HASMCs in 12-well plates were co-transfected with a firefly luciferase gene tagged with renilla luciferase and pGL4.32-NF-κB using the FuGENE HD reagent (Invitrogen, Carlsbad, CA, USA). Medium was replaced with fresh medium after 6 h. At 24 h post-transfection, cells were stimulated with TNF-α (10 ng/mL) and various concentrations of *PAE* (50, 100, 250 μg/mL). Luciferase activity was assayed 24 h later using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.5. Animals and diets

Male ApoE KO mice (6 weeks of age; Jackson Laboratory, Bar Harbor, ME) having a C57BL/6N genetic background were housed under diurnal lighting conditions and allowed food and tap water ad libitum. All experimental protocols involving the use of animals were conducted in accordance with National Institutes Health (NIH) guidelines and approved by the committee on Animal care of the KIOM. Six-week-old ApoE KO mice fed on a Western diet (45% of total calories from fat, 0.15% cholesterol; Research Diet, New Brunswick, NJ) were divided into five groups: Control ($n=8$), Vehicle ($n=8$), PAE 50 mg/kg treatment ($n=10$), PAE 100 mg/kg ($n=10$), and Atorvastatin 10 mg/kg ($n=9$). PAE (50 or 100 mg/kg), statin (Atorvastatin 10 mg/kg), or an equal volume of saline (vehicle) was given orally each day for 12 weeks with a Western diet.

2.6. Body and adipose tissue weights

Adipose tissue (subcutaneous and retroperitoneal) were removed from mice and weighed immediately. Body weights were measured once per week.

2.7. Serum measurements

Blood was collected from the aorta under light anesthesia and stored on ice for 30 min before centrifugation (13,000 rpm, 4 °C, 10 min). The serum was separated and kept at −80 °C until assayed. Serum levels of total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were analyzed with an automatic analyzer.

2.8. Blood pressure measurements

Blood pressure was monitored in the Control and treatment groups (HFD, *PAE* 50 or 100 mg/kg and Atorvastatin 10 mg/kg) with a non-invasive tail cuff CODA system (Kent Scientific, Torrington, CT, USA) (Daugherty et al., 2009).

2.9. Histology

White adipose tissues (subcutaneous and retroperitoneal) and the aorta were removed from the mice and weighed immediately. To stain adipocytes, adipose tissues and aorta were fixed in 10% formalin solution for 1 day and then embedded in paraffin wax. All tissues were cut to a thickness of 6 μm and stained with hematoxylin and eosin. All samples were examined using a light microscope (Carl Zeiss, Jena, Germany) (Sung et al., 2012).

2.10. Western blotting

Proteins from HASMCs or aorta tissues were isolated according to standard techniques, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Blots were probed for VCAM-1, NF-κB (p65), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ICAM-1, E-selectin, extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38 (Cell Signaling Technology, Beverly, MA, USA) followed by incubation with secondary antibody (Cell Signaling) conjugated with horseradish peroxidase. The intensity of chemiluminescence was measured with an ImageQuant LAS 4000 apparatus (GE Healthcare Life Sciences, Buckinghamshire, UK).