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Original Research

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Purple perilla frutescens extracts containing α-asarone inhibit inflammatory atheroma formation and promote hepatic HDL cholesterol uptake in dyslipidemic apoE-deficient mice

Sin-Hye Park [1], **Young Eun Sim** [1], **Min-Kyung Kang** [2], **Dong Yeon Kim** [2], **Il-Jun Kang** [1], **Soon Sung Lim** [1], **and Young-Hee Kang** [1§]

[1]Department of Food Science and Nutrition and Korean Institute of Nutrition, Hallym University, Chuncheon 24252, Korea
[2]Department of Food and Nutrition, Andong National University, Andong 36729, Korea

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[§]Corresponding Author:
Young-Hee Kang
Department of Food Science and Nutrition and Korean Institute of Nutrition, Hallym University, 1 Hallymdaehak-gil, Chuncheon 24252, Korea.
Tel. +82-33-248-2132
Fax. +82-33-254-1475
Email. yhkang@hallym.ac.kr

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ORCID iDs
Sin-Hye Park
https://orcid.org/0000-0003-1656-185X
Young Eun Sim
https://orcid.org/0000-0002-1521-2896
Min-Kyung Kang
https://orcid.org/0000-0002-9461-9220
Dong Yeon Kim
https://orcid.org/0000-0001-9177-2935

ABSTRACT

BACKGROUND/OBJECTIVES: Dyslipidemia causes metabolic disorders such as atherosclerosis and fatty liver syndrome due to abnormally high blood lipids. Purple perilla frutescens extract (PPE) possesses various bioactive compounds such as α-asarone, chlorogenic acid and rosmarinic acid. This study examined whether PPE and α-asarone improved dyslipidemia-associated inflammation and inhibited atheroma formation in apolipoprotein E (apoE)-deficient mice, an experimental animal model of atherosclerosis.
MATERIALS/METHODS: ApoE-deficient mice were fed on high cholesterol-diet (Paigen's diet) and orally administrated with 10–20 mg/kg PPE and α-asarone for 10 wk.
RESULTS: The Paigen's diet reduced body weight gain in apoE-deficient mice, which was not restored by PPE or α-asarone. PPE or α-asarone improved the plasma lipid profiles in Paigen's diet-fed apoE-deficient mice, and despite a small increase in high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein (LDL)-cholesterol, and very LDL were significantly reduced. Paigen's diet-induced systemic inflammation was reduced in PPE or α-asarone-treated apoE-deficient mice. Supplying PPE or α-asarone to mice lacking apoE suppressed aorta atherogenesis induced by atherogenic diet. PPE or α-asarone diminished aorta accumulation of CD68- and/or F4/80-positive macrophages induced by atherogenic diet in apoE-deficient mice. Treatment of apoE-deficient mice with PPE and α-asarone resulted in a significant decrease in plasma cholesteryl ester transfer protein level and an increase in lecithin:cholesterol acyltransferase reduced by supply of Paigen's diet. Supplementation of PPE and α-asarone enhanced the transcription of hepatic apoA1 and SR-B1 reduced by Paigen's diet in apoE-deficient mice.
CONCLUSIONS: α-Asarone in PPE inhibited inflammation-associated atheroma formation and promoted hepatic HDL-C trafficking in dyslipidemic mice.

Keywords: Alpha-asarone; apolipoproteins E; atheroma; high density lipoprotein, purple perilla frutescens extract

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Il-Jun Kang
https://orcid.org/0000-0003-0314-5195
Soon Sung Lim
https://orcid.org/0000-0003-4548-1285
Young-Hee Kang
https://orcid.org/0000-0003-2039-7968

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Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Park SH, Kang YH; Data curation: Park SH, Sim YE, Kim DY; Formal analysis: Park SH, Kang MK, Lim SS; Writing - original draft: Park SH, Kang YH; Writing - review & editing: Kang IJ, Kang YH.

INTRODUCTION

Atherogenic dyslipidemia has emerged as a major risk factor for myocardial infarction and cardiovascular diseases (CVD) with severe complications [1]. Atherogenic dyslipidemia comprises a triad of increased low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG), and decreased high-density lipoprotein cholesterol (HDL-C), and is known to be caused by the imbalance of these lipids [1,2]. Atherogenic dyslipidemia is enhanced by fat-rich diet, smoking, physical inactivity, obesity and type 2 diabetes mellitus [1]. High saturated fat intake increases CVD risk in phenotype B individuals with small LDL particles, accompanying increases in total cholesterol (TC) and LDL-C [3]. Atherogenic dyslipidemia is mainly controlled and managed in an unconvincing manner due to available therapeutic limitations [4]. Evidence is increasing to support that a mixture of lipid-lowering agents such as statins (HMG-CoA reductase inhibitors) and other drugs, reduces CVD and mortality in patients with dyslipidemia [5,6]. In addition, the approved non-statin drugs such as HDL particles, niacin, cholesteryl ester transfer protein (CETP) inhibitors and LDL-C-lowering agents are ready to take on novel roles in managing dyslipidemia and hypercholesterolemia [5,6].

Apolipoprotein (apo) E is present as a part of chylomicron remnants, very low-density lipoprotein (VLDL), intermediate-density lipoprotein and some HDL [7]. In peripheral tissues apoE primarily produced by the liver and macrophages, interacts significantly with the LDL receptor and mediates the normal catabolism of TG-rich lipoproteins and cholesterol metabolism [8,9]. Accordingly, apoE deficiency or abnormality comes up a series of pathological conditions including dyslipidemia, hypercholesterolemia, atherosclerosis and Alzheimer's disease [10]. The apoE deletion results in increased hepatic cholesterol content, decreased HDL, and decreased hepatic HMG-CoA reductase activity [10]. On the other hand, apoE plays a crucial role in regulation of lipid metabolism through influencing the activity of hepatic lipase and CETP [8,9]. Also, apoE promotes cholesterol efflux from lipid-loaded macrophages in the artery wall, which is thought to be atheroprotective [9,11]. ApoE, rather than apoA1, may activate lecithin:cholesterol acyltransferase (LCAT) on large HDL particles to promote esterification of effluxed free cholesterol [11]. Nevertheless, the precise mechanism of action of highly sensitive effects of apoE is not yet clear.

Despite development of the prevention and treatment of dyslipidemia, the use of medications to treat dyslipidemia is limited to chemicals. Several studies review conventional natural therapies for metabolic abnormalities, including atherogenic dyslipidemia, obesity, and insulin resistance [12,13]. Naturally-occurring bioactive compounds are highly efficient, mostly safe and very-well tolerated [13,14]. Purple perilla frutescens (**Fig. 1A**) is an edible plant broadly growing in Eastern Asia including China and Japan as well as Korea. Purple perilla frutescens has several health benefits to treat depression-related disease and asthma [15,16]. Our previous studies have shown that the extracts of purple perilla frutescens (PPE) enhance cholesterol efflux from oxidized LDL-exposed macrophages and antagonize macrophage endoplasmic reticulum stress in lipid-laden macrophages [17,18]. In addition, the constituents to inhibit aldose reductase and xanthine oxidase are identified in PPE [19,20]. Furthermore, one investigation shows that α-asarone inhibits HMG-CoA reductase, lowers serum LDL-C levels and reduces biliary cholesterol saturation index in hypercholesterolemic rats [21]. Based on these findings, the current study attempted to determine that PPE blocked dyslipidemia-induced inflammation and inhibited atheroma formation in high cholesterol diet-fed apoE-deficient mice. In addition, this study investigated whether α-asarone (**Fig. 1A**), a bioactive component in PPE inhibited atherosclerotic abnormalities and dyslipidemia in mice lacking apoE [22].

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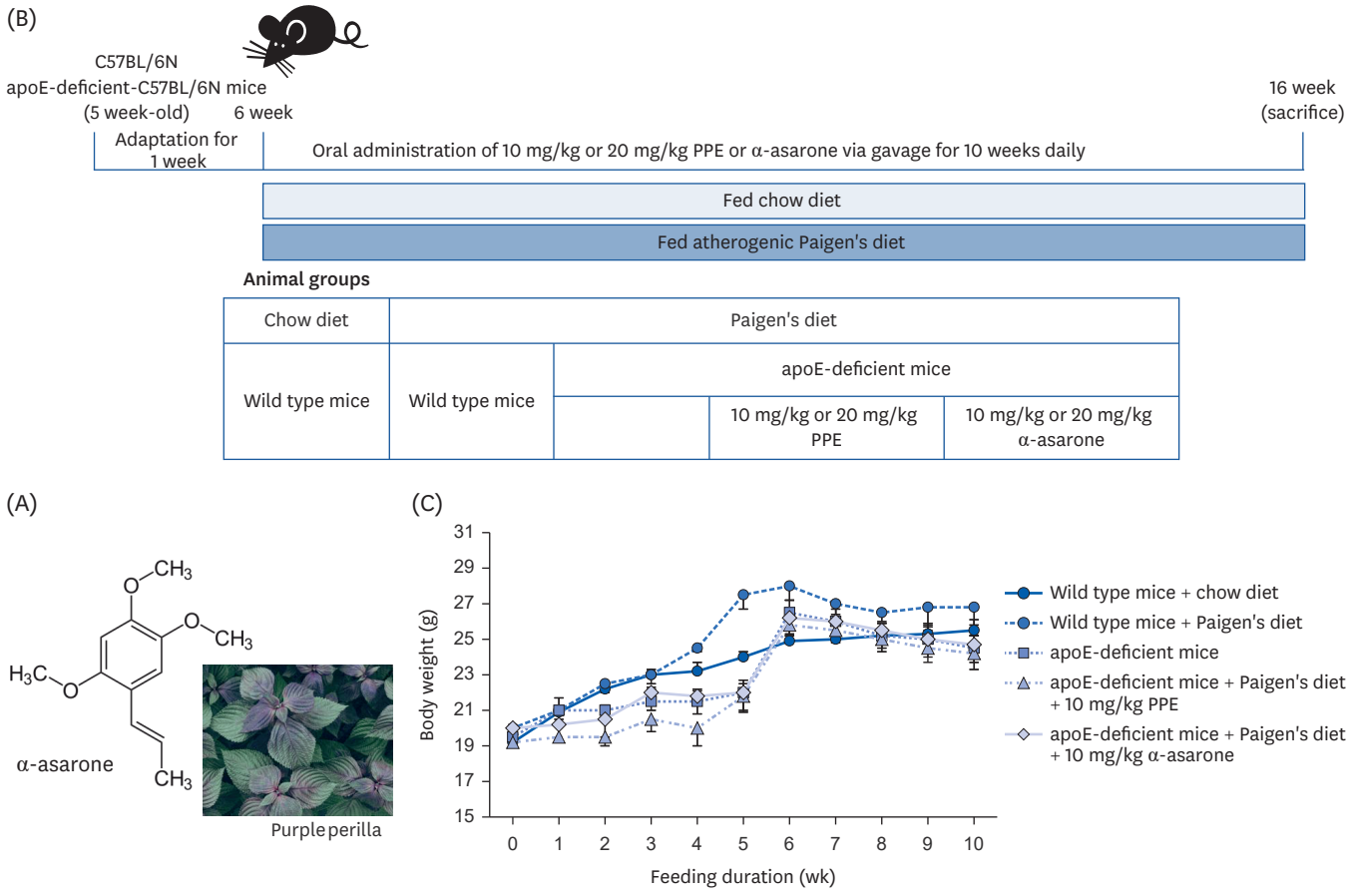

Fig. 1. Chemical structure of α-asarone (A), animal experimental design and grouping (B), and change of BWs during feeding (C). Wild type and homozygous apoE-deficient C57BL/6N mice (5 wk of age) were fed either chow diet or atherogenic Paigen's diet. Atherogenic Paigen's diet-fed apoE-deficient mice were divided into 5 subgroups. These apoE-deficient mice received 10–20 mg/kg PPE or 10–20 mg/kg α-asarone via gavage daily for 10 wk. The animal BW was measured at the beginning of the experiment and at 1 wk intervals for 10 wk. Values in curved linear graphs were expressed as mean ± SEM (n = 8–10).
apoE, apolipoprotein E; PPE, purple perilla frutescens extract; BW, body weight.

MATERIALS AND METHODS

Animals and diets

Homozygous apoE-deficient and wild type C57BL/6 mice (5 wk of age) were obtained from Japan Shizuoka Laboratory Center and the males used for this study. Mice were housed individually in wire-bottomed cages and kept on a 12 h light/12 h dark cycle at 20–25°C with 60% relative humidity under specific pathogen-free conditions. The animal experimental design and grouping were described in **Fig. 1B**. The WT mice were fed with chow or atherogenic diet (Paigen's diet; including 1.25% cholesterol) for 10 wk supplied by Research Diets, Inc. (New Brunswick, NJ, USA). The apoE-deficient mice were divided into 5 groups. One group was fed on atherogenic diet, and the other groups were fed on 10 mg/kg/body weight (BW) PPE, 20 mg/kg/BW PPE, 10 mg/kg/BW α-asarone, or 20 mg/kg/BW α-asarone during same experimental periods. The animals were allowed to acclimatize for a week before beginning the experiments. All animal experiments were performed in accordance with the University's Guidelines for the Care and Use of Laboratory Animals approved by the Committee on Animal Experimentation of Hallym University (hallym R2014-40). No mice were dead and no apparent signs of exhaustion were observed during the experimental period.

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The PPE preparation and the identification of α-asarone were described in our previous studies [20,21]. Purple perilla was obtained from a local market in Chuncheon, and α-asarone was purchased from Cayman Chemical (Ann Arbor, MI, USA). Dried leaves of Perilla frutescens (2 kg) were extracted 3 times with 99.5% methanol for 5 h. The solvent was evaporated under reduced pressure below 45°C to give a methanol extract. The extract was suspended in distilled water and partitioned with n-hexane, methylene chloride, ethyl acetate, n-butanol, and H_2O. A portion of the n-hexan fraction (purple perilla extract, PPE) was purified by chromatography on silica gel eluted with chloroform and increasing proportion methanol (10:0–9:1) to yield eleven parts (part 1–10). The part 5 (0.46 g) showing the most potent activity was further purified via recrystallization to yield compound 1 (76 mg). The ^{1}H-NMR, ^{13}C-NMR, ESI-MS, and UV data confirmed compound 1 as α-asarone.

Measurements of food intake and weighs of body and organs

Food intake, BW and organ weight were measured in chow diet- or atherogenic diet-fed mice every week during the 10 wk-supplementation of PPE and α-asarone. Pre-weighed food was supplied in a standard stainless steel hopper. The amount of food left over was measured, including what was on our floor or spilled on the plastic sheet placed under each cage.

At the end of the experiment, all the mice were sacrificed under zoletin/lumphoon anesthesia. Blood samples were collected from the eye into EDTA-coated tubes. Plasma samples were obtained by at 3,000 rpm for 10 min and stored at –70^{0}C. The organs were washed with physiological saline by direct injection in the heart left ventricule. Collected aortas were immediately frozen in liquid N2 until analysis, and conducted to fixation with formaldehyde for the immunohistochemistry analysis. The collected livers were homogenized for the RNA collection.

Measurements of plasma lipids

After blood was centrifuged, plasma was used to measure lipid profile and contents. TC, TG and HDL-C were measured using enzymatic assays (Asan Pharmaceuticals, Hwasung, Korea). LDL-C was determined by using the formula, LDL = TC − (HDL − TG/5). In addition, VLDL was calculated by the formula, VLDL = TG/5. Based on these values, the atherosclerosis index (AI) was obtained by using the formula, AI = (TC − HDL-C)/HDL-C.

Enzyme-linked immunosorbent assay (ELISA)

The plasma levels of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-1β, CETP, phospholipid transfer protein (PLTP), and LCAT were examined by using sandwich-type ELISA kits (USCN, Wuhan, China; R&D systems, Minneapolis, MN, USA; MyBioSource, San Diego, CA, USA), according to the manufacturer's instructions. After reacting plasma samples on plate wells pre-coated with a biotinylated antibody of MCP-1, IL-1β, CETP, PLTP, or LCAT, an avidin-conjugated horseradish peroxidase (HRP) was added to microplate wells. The TMB substrate was added to wells for detecting color change and the enzyme-substrate reaction is terminated by the addition of 3 N sulfuric acid. The changed color was measured by spectrophotometry at λ = 450 nm.

Aortic atheroma formation

Aortic atheroma formation was assessed by staining lipid deposition in the aortas. The aortas were dissected and fixed with 4% paraformaldehyde solution overnight. Subsequently, fixed aorta tissues were soaked with 30% sucrose for dehydration. The aorta tissues were embedded with OCT compound and cut into 5 μm thickness on a glass slides by using a

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microtome. To identify the atheroma in the aortas, the sections of aorta tissues were stained by hematoxylin and oil red O. The stained samples were observed with microscopes.

Immunohistochemical analysis

For the immunohistochemical staining, aortas were obtained at the end of the experiments and fixed in 10% buffered formalin. The 5 μm-thick sections of frozen aorta tissues were dried on the air at room temperature for 20 min, rehydrated with PBS for 10 min and quenched endogenous peroxidase activity with 3% H_2O_2 solution for 10 min. Subsequently, tissues were incubated with 2% serum blocking buffer for 1 h. The tissue sections were incubated with a primary antibody of CD68 (Santa Cruz Biotechnol., Heidelberg, Germany) and F4/80 (Abcam, Boston, MA, USA) overnight and HRP-conjugated anti-mouse IgG for 1 h. For the CD68 visualization, the sections were visualized with 3,3′-diaminobenzidine to produce a brown staining, being counterstained with hematoxylin. For the measurement of F4/80 expression, the sections were visualized with vector NovaRED substrate (Vector Laboratories, Newark, CA, USA) to produce a red staining. The stained tissue sections were examined using an optical Axiomager microscope system (Zeiss, Göttingen, Germany) and 5 images (400×) were taken for each section.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The liver tissues were homogenized with Trizol, and then centrifuged at 12,000 rpm for 20 min. Supernatants were collected to new tube and isopropanol was added to for the RNA precipitation. After centrifugation, pellets were RNA and quantified with spectrophotometry at λ = 260 nm. For the cDNA, same amount of RNA (5 μg) were conducted with 200 units of reverse transcriptase at 42°C for 50 min and at 70°C for 15 min. The polymerase chain reaction (PCR) was conducted using mRNA transcripts of mouse apoA-1 (forward primer: 5′-TCC-CAG-AAG-TCC-CGA-GTC-AA-3′, reverse primer: 5′-AGC-AAG-ATG-AAC-CCC-AGT-CC-3′, product size:220 bp), scavenger receptor (SR)-B1 (forward primer: 5′-ATG-GGC-CAG-CGT-GCT-TTT-ATG-A-3′, reverse primer: 5′-AAC-CAC-AGC-AAC-GGC-AGA-ACT-A-3′, product size:752 bp), β-actin (forward primer: 5′-GAC-TAC-CTC-ATG-AAG-ATC-3′, reverse primer: 5′-GAT-CCA-CAT-CTG-CTG-GAA-3′, product size:500 bp), and GAPDH (forward primer:5′-AAC-TTT-GGC-ATT-GTG-GAA-GGG-3′, reverse primer: 5′-GAC-ACA-TTG-GGG-GTA-GGA-ACA-C-3′, product size:224 bp) with an addition of 25 μL of 10 mM Tris-HCl (pH 9.0) containing 25 mM $MgCl_2$, 10 mM deoxynucleotide triphosphate and 5 units of Taq DNA polymerase. Each cycle (35 cycles) consisted of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C, and the final extension was for 10 min at 72°C. After thermocycling and electrophoresis of the PCR products (20 μL) on 1% agarose gel containing 0.1% ethidium bromide, the bands were visualized using a TFX-20M model-UV transilluminator (Vilber-Lourmat, Marene-la-Vallee, France) and gel photographs were obtained.

Statistical analysis

The data are presented as means ± SEM. Statistical analyses were carried out employing Statistical Analysis Systems statistical software package (SAS Institute Inc., Cary, NC, USA). Statistical significance was determined by 1-way analysis of variance, followed by Duncan range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

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RESULTS

Weights of body and organs

The BW of wild type mice fed chow diet increased throughout the feeding duration (**Fig. 1C**). However, the BW of wild type mice fed atherogenic diet highly increased up to 5th wk and thereafter the increase declined (**Fig. 1C**). Thus, the BW gain was not influenced in atherogenic Paigen's diet-fed wild type mice, as with food intake (**Table 1**). However, despite a similar amount of food intake, the atherogenic diet reduced weight gain in mice that lacked apoE. When PPE or α-asarone was administered to apoE-deficient mice, the BW gain and the food efficiency ratio were not improved (**Fig. 1C** and **Table 1**). BW and food efficiency ratio has dropped dramatically in apoE-knockout mice treated with 20 mg/kg α-asarone.

The wet weights of the heart and liver of atherogenic Paigen's diet-fed mice were much higher than those of chow diet-fed mice, while the kidney of atherogenic apoE-knockout mice was relatively lighter (**Table 1**). The weights of the heart and liver were reduced by supplementing 10 mg/kg PPE and α-asarone.

Plasma lipid profile

The plasma levels of TC and LDL-C of Paigen's diet-fed wild mice increased significantly, and these levels were further elevated (≤ 10-fold) in atherogenic diet-fed apoE-deficient mice (**Table 2**). In addition, the plasma levels of TG and VLDL were much higher in atherogenic diet-fed apoE-knockout mice, compared to those of wild mice. Conversely, the plasma level of HDL-C declined greatly (≤ 10-fold) in atherogenic diet-fed apoE mice, compared to those of wild mice (**Table 2**). When apoE-deficient mice were fed with atherogenic diet containing PPE or α-asarone, the plasma lipid profiles were improved, with considerable reduction in TC, TG, LDL-C, and VLDL despite a small increase in HDL (**Table 2**). As a result, the AI declined in a positive direction in PPE or α-asarone-supplied apoE-knockout mice.

Effects of PPE and α-asarone on inflammatory formation of aortic atheroma

The transgenic apoE-deficient mice develop severe hypercholesterolemia due to aberrant clearance of atherogenic lipid particles from the circulation [23]. This study employed apoE-knockout mice with an atherosclerosis-susceptible C57BL/6N genetic background, as an

Table 1. BW, food intake and organ weight in mice fed on chow diet or Paigen's diet

Each group (n = 5–9)	Chow diet	Atherogenic Paigen's diet					
	Wild type mice	Wild type mice	ApoE-deficient mice				
				10 mg/kg PPE	20 mg/kg PPE	10 mg/kg α-asarone	20 mg/kg α-asarone
BW (g)							
Initial	20.10 ± 0.28^{a}	21.30 ± 0.26^{a}	20.78 ± 0.52^{a}	19.88 ± 0.30^{a}	20.83 ± 0.60^{a}	20.00 ± 0.30^{a}	21.38 ± 0.32^{a}
Final (10 wk)	25.50 ± 0.37^{ab}	26.80 ± 0.39^{a}	24.33 ± 0.67^{b}	23.63 ± 0.60^{b}	24.00 ± 0.26^{b}	24.30 ± 0.40^{b}	22.50 ± 0.19^{c}
Δweight	5.40 ± 0.50^{a}	5.50 ± 0.34^{a}	4.136 ± 0.35^{b}	3.75 ± 0.56^{b}	3.17 ± 0.60^{b}	4.30 ± 0.56^{b}	1.13 ± 0.23^{c}
ADG	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	0.05 ± 0.02^{b}	0.06 ± 0.01^{b}	0.02 ± 0.01^{c}
Food intake							
ADFI	3.87 ± 0.02^{a}	3.88 ± 0.03^{a}	3.90 ± 0.03^{a}	3.84 ± 0.05^{a}	3.71 ± 0.05^{b}	3.69 ± 0.05^{b}	3.63 ± 0.03^{b}
FER	1.40 ± 0.13^{a}	1.42 ± 0.09^{a}	0.95 ± 0.14^{b}	0.98 ± 0.1^{b}	0.84 ± 0.16^{b}	1.17 ± 0.15^{b}	0.31 ± 0.06^{c}
Organ weight (g)							
Heart	0.17 ± 0.01^{b}	0.27 ± 0.01^{a}	0.22 ± 0.01^{a}	0.18 ± 0.01^{b}	0.23 ± 0.01^{a}	0.19 ± 0.01^{b}	0.28 ± 0.01^{a}
Liver	0.94 ± 0.03^{c}	1.72 ± 0.03^{a}	1.78 ± 0.13^{a}	1.24 ± 0.03^{b}	1.60 ± 0.07^{a}	1.31 ± 0.04^{b}	1.72 ± 0.17^{a}
Kidney	0.43 ± 0.01^{a}	0.43 ± 0.01^{a}	0.39 ± 0.01^{b}	0.31 ± 0.01^{b}	0.33 ± 0.02^{b}	0.32 ± 0.01^{b}	0.38 ± 0.01^{b}

Wild type mice and apoE-deficient mice were fed with chow diet or atherogenic Paigen's diet for 10 wk with and without 10–20 mg/kg PPE or α-asarone. Wild type mice were fed chow diet as control mice. Statistical evaluation was done with Duncan range test for multiple comparisons after 1-way analysis of variance. Respective values (mean ± SEM) not sharing a small alphabetical letter in the same row are different at $P < 0.05$.
BW, body weight; ADG, average daily gain; ADFI, average daily food intake; FER, food efficiency ratio; apoE, apolipoprotein E; PPE, purple perilla frutescens extract.

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Table 2. Plasma lipid profiles in mice fed on chow diet or Paigen's diet

Each group (n = 5–9)	Chow diet	Atherogenic Paigen's diet					
	Wild type mice	Wild type mice	ApoE-knockout mice				
				10 mg/kg PPE	20 mg/kg PPE	10 mg/kg α-asarone	20 mg/kg α-asarone
TC	115.97 ± 3.41^{e}	192.01 ± 7.53^{d}	1,871.50 ± 154.49^{a}	1,336.61 ± 84.08^{c}	1,270.61 ± 62.22^{c}	1,514.16 ± 90.40^{b}	1,572.38 ± 87.46^{b}
TG	39.94 ± 2.11^{d}	47.97 ± 4.34^{c}	93.13 ± 4.58^{a}	86.83 ± 11.22^{a,b}	76.40 ± 9.542^{b}	73.078 ± 4.018^{b}	71.12 ± 6.32^{b}
HDL-C	58.45 ± 6.17^{a}	48.98 ± 5.41^{a}	6.13 ± 0.82^{c}	6.05 ± 1.31^{c}	8.88 ± 2.78^{b,c}	7.822 ± 1.148^{c}	11.48 ± 2.11^{b}
LDL-C	49.53 ± 6.30^{e}	133.43 ± 8.69^{d}	1,846.74 ± 154.20^{a}	1,313.19 ± 85.43^{c}	1,246.64 ± 59.63^{c}	1,491.72 ± 90.70^{b}	1,546.68 ± 87.45^{b}
VLDL	7.99 ± 0.42^{d}	9.59 ± 0.87^{c}	18.63 ± 0.92^{a}	17.37 ± 2.24^{a,b}	15.28 ± 1.91^{b}	14.62 ± 0.80^{b}	14.23 ± 1.26^{b}
AI	1.19 ± 0.23^{e}	3.46 ± 0.60^{d}	351.65 ± 53.58^{a}	288.82 ± 46.62^{a,b}	235.90 ± 83.81^{b}	231.48 ± 38.88^{b}	191.53 ± 32.16^{c}

Wild type mice and apoE-deficient mice were fed with chow diet or atherogenic Paigen's diet for 10 wk with and without 10–20 mg/kg PPE or α-asarone. Wild type mice were fed chow diet as control mice. Blood lipid contents were analyzed by using commercial detection kits. LDL-C = TC − HDL-C − (TG/5). VLDL = TG/5. AI = (TC − HDL-C)/HDL-C. Statistical significance was determined with Duncan range test for multiple comparisons after 1-way analysis of variance. Respective values (mean ± SEM) not sharing a small alphabetical letter in the same row are different at $P < 0.05$.
apoE, apolipoprotein E; TC, total cholesterol; TG, triglyceride, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein; AI, atherosclerosis index; PPE, purple perilla frutescens extract.

experimental animal model of atherosclerosis. The current study examined whether chronic supply of atherogenic diet to apoE-deficient mice induced systemic inflammation, which was inhibited by treating PPE and α-asarone. The wild type mice exposed to a Paigen's diet for 10 wks resulted in a significant increase in plasma MCP-1 level (**Fig. 2A**). In addition, plasma IL-1β level slightly increased in atherogenic diet-fed wild type mice (**Fig. 2B**). However, the 10 wk-feeding of Paigen's diet-alone to mice lacking apoE highly elevated the plasma levels of these inflammatory mediators. When PPE or α-asarone was treated to apoE-lacking mice, the systemic inflammation was alleviated (**Fig. 2A and B**).

The present study examined whether PPE and α-asarone reduced atheroma formation in atherogenic apoE-knockout mice, evidenced by oil red O staining. When apoE-deficient mice were fed a Paigen's diet for 10 wks, severe atheroma was developed in aortas, compared to those of wild type mice fed the atherogenic diet (**Fig. 2C**). In contrast, the aortic atheroma was highly suppressed by supplementing 20 mg/kg PPE or 20 mg/kg α-asarone to these mice. Therefore, PPE containing α-asarone may be a therapeutic agent against inflammatory atheroma formation due to hypercholesterolemia.

Inhibition of inflammatory cell infiltration by PPE and α-asarone

Macrophages are identified in murine tissues using a small range of markers such as F4/80, CD68 and CD11b [24]. This study attempted to reveal that PPE or α-asarone inhibited the infiltration of foamy macrophages in aortas of apoE-deficient mice. There was no induction of CD68 and F4/80 in the aortas of wild type mice, evidenced by immunohistochemical staining (**Fig. 3**). The staining of CD68 and F4/80 was enhanced in apoE-knockout mice fed Paigen's diet, indicating that numerous macrophages were infiltrated to foamy aortas (arrows). When the apoE-lacking mice were supplemented with PPE or α-asarone, the accumulation of CD68- and/or F4/80-positive macrophages was diminished (**Fig. 3**).

Effects of PPE and α-asarone on plasma levels of lipid transfer proteins

Atherosclerosis-prone apoE-knockout mice accumulate cholesterol ester-enriched lipids in the blood due to poor lipoprotein clearance [25]. This study investigated that PPE and α-asarone influenced the plasma levels of lipid transfer proteins involved in the reverse cholesterol transport (RCT). The Paigen's diet feeding tended to increase plasma levels of CETP and PLTP in wild type mice (**Fig. 4A and B**). In addition, the atherogenic feeding markedly enhanced the levels of these transfer proteins in apoE-deficient mice. The administration of PPE and α-asarone to atherogenic diet-fed apoE-lacking mice diminished

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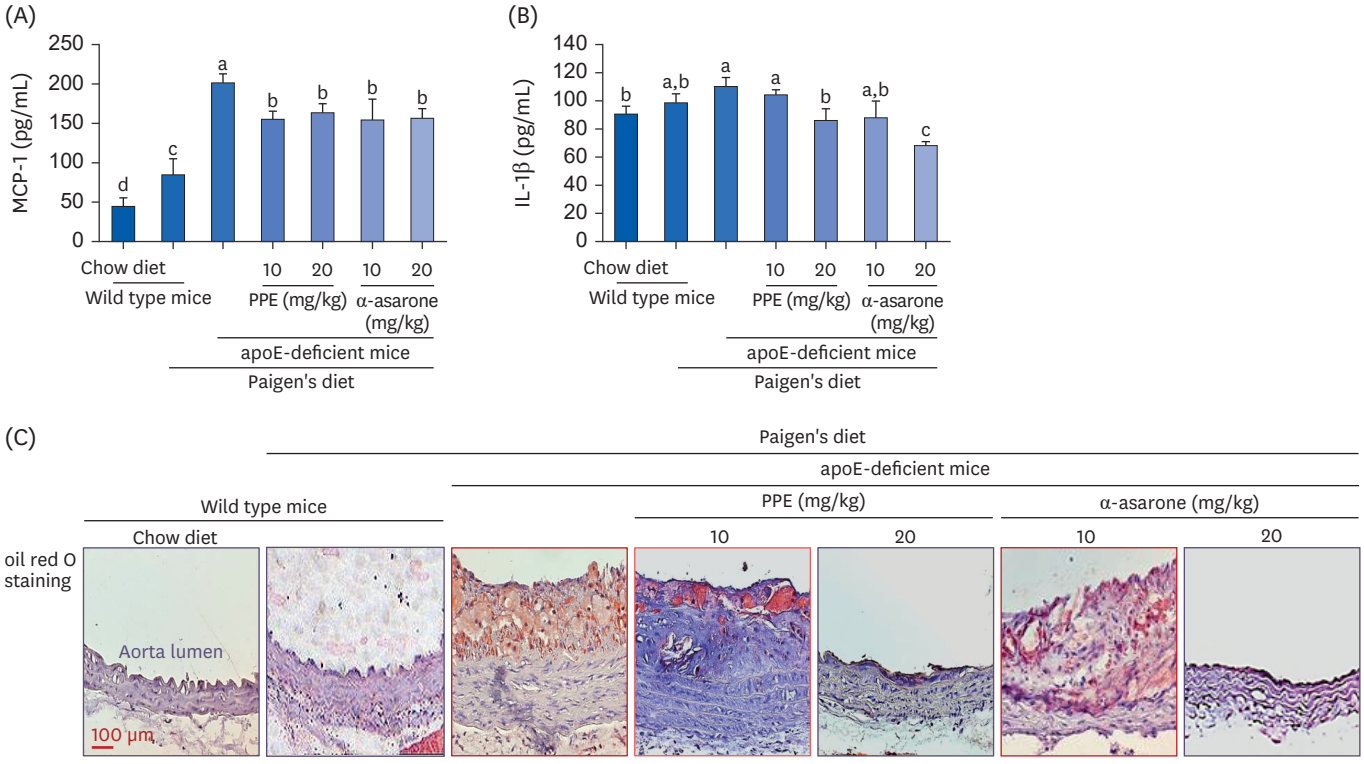

Fig. 2. Effects of PPE and α-asarone on circulatory inflammation (A, B) and atheroma formation (C) in apoE-deficient mice fed atherogenic Paigen's diet. Wild type and homozygous apoE-knockout C57BL/6N mice (5 wk of age) were fed either chow diet or atherogenic Paigen's diet. Atherogenic Paigen's diet-fed apoE-deficient mice were divided into 5 subgroups. These apoE-deficient mice received 10–20 mg/kg PPE or 10–20 mg/kg α-asarone via gavage daily for 10 wk. For the measurements of systemic inflammation, plasma samples were analyzed with commercial enzyme-linked immunosorbent assay kits of MCP-1 (A) and IL-1β (B). Respective values (mean ± SEM, n = 7) in bar graphs not sharing a small alphabetical letter are different at $P < 0.05$. For the observation of atheroma in the aorta, aorta cut-tissues were stained with oil red O, and counterstained with hematoxylin (C). Scale bar represents 100 μm. The stained tissues were observed with microscopes (n = 4).
MCP-1, monocyte chemoattractant protein-1; IL-1β, interleukin-1β; apoE, apolipoprotein E; PPE, purple perilla frutescens extract; BW, body weight.

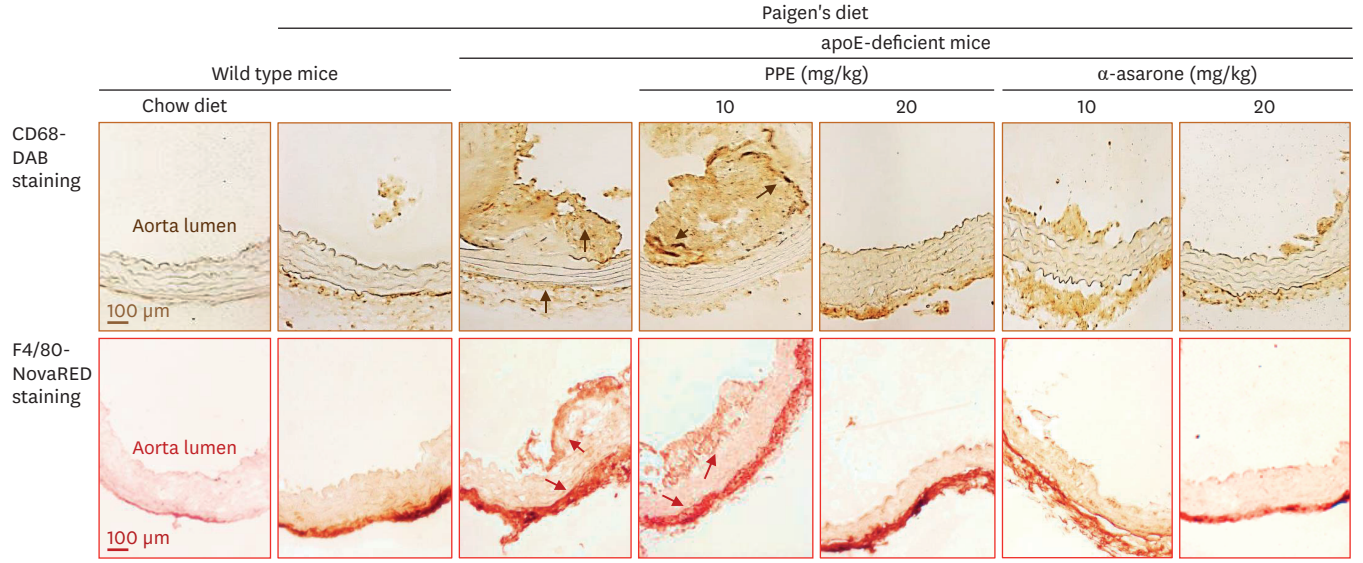

Fig. 3. Inhibition of inflammatory cell trafficking by PPE and α-asarone. Wild type and homozygous apoE-knockout C57BL/6N mice (5 wk of age) were fed either chow diet or atherogenic Paigen's diet. Atherogenic Paigen's diet-fed apoE-deficient mice were divided into 5 subgroups. These apoE-deficient mice received 10–20 mg/kg PPE or α-asarone via gavage daily for 10 wk. For the observation of macrophage infiltration into the aorta wall, aorta cut-tissues were immunohistochemically stained with an antibody against CD68 and F4/80. For the visualization of CD68 and F4/80, the tissue sections were stained with substrates of DAB (brown precipitate) and NovaRED (red precipitate). Scale bars represent 100 μm. Each photograph is representative of at least 4 animals. The stained tissues were observed with fluorescent microscopes.
PPE, purple perilla frutescens extract; apoE, apolipoprotein E; DAB, 3,3′-diaminobenzidine

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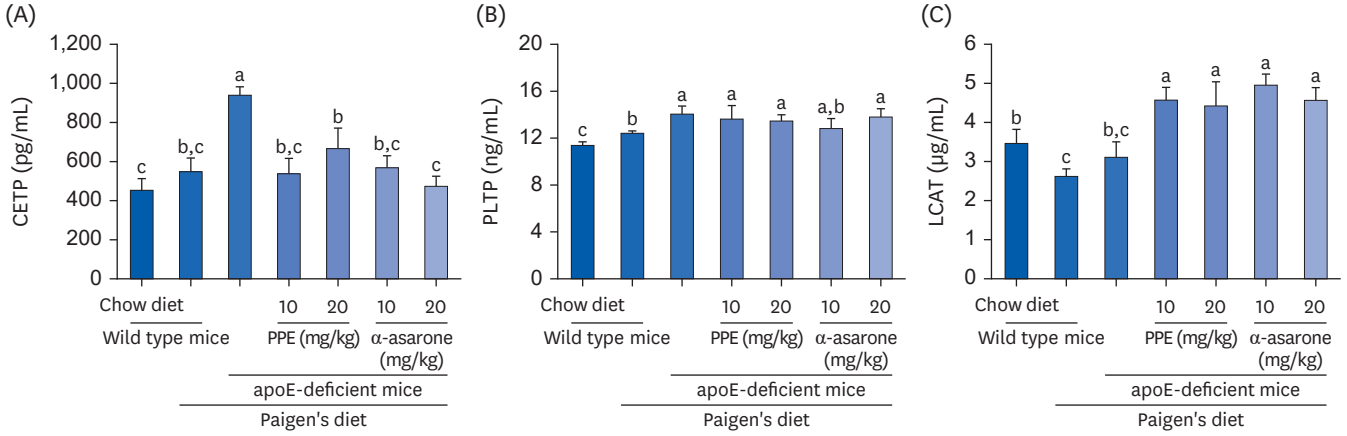

Fig. 4. Regulatory effects of PPE and α-asarone on the levels of lipid transfer proteins in atherogenesis. Wild type and homozygous apoE-knockout C57BL/6N mice (5 wk of age) were fed either chow diet or atherogenic Paigen's diet. Atherogenic Paigen's diet-fed apoE-deficient mice were divided into 5 subgroups. These apoE-deficient mice received 10–20 mg/kg PPE or 10–20 mg/kg α-asarone via gavage daily for 10 wk. Plasma levels of CETP, PLTP, and LCAT were analyzed by commercial enzyme-linked immunosorbent assay kits of CETP (A), PLTP (B), and LCAT (C). Respective values (mean ± SEM, n = 7) in bar graphs not sharing a small alphabetical letter are different at $P < 0.05$.
CETP, cholesterol ester transfer protein; PPE, purple perilla frutescens extract; apoE, apolipoprotein E; PLTP, phospholipid transfer protein; LCAT, lecithin:cholesterol acyltransferase.

plasma level of CETP responsible for cholesteryl ester transportation from HDL to TG-rich lipoproteins (**Fig. 4A**). However, the plasma PLTP level was not changed (**Fig. 4B**). On the other hand, the plasma LCAT level was down-regulated by Paigen's diet feeding in wild type mice and apoE-deficient mice (**Fig. 4C**). Treating apoE-deficient mice with PPE and α-asarone highly elevated the LCAT level. Accordingly, PPE and α-asarone may promote RCT through affecting plasma levels of CETP and LCAT.

Elevation of hepatic HDL-C uptake by PPE and α-asarone

Endogenous apoA1 promoter can drive sustained expression of apoE, highly reducing plasma lipids in a model of hypercholesterolemia [26]. This study examined whether PPE and α-asarone restored the transcription of apoA1, a HDL component, evidenced by quantitative RT-PCR assay. The Paigen's diet feeding induced a small reduction of apoA1 transcription in wild type mice. However, the atherogenic diet feeding greatly reduced the apoA1 transcription in mice lacking apoE (**Fig. 5**). On the contrary, oral administration of PPE and α-asarone enhanced the transcription of hepatic apoA1 in apoE-deficient mice (**Fig. 5**). It should be noted that the effects of α-asarone was much greater. Accordingly, α-asarone in PPE may promote hepatic ApoA1 synthesis and secretion in atherosclerosis-prone apoE-deficient mice.

Cholesterol from peripheral tissues, carried by HDL, is metabolized in the liver after uptake by the HDL receptor, SR-B1 [27]. This study further investigated that PPE or α-asarone improved the transcriptional expression of hepatic SR-B1 in atherogenic diet-fed apoE-knockout mice. The RT-PCR analysis showed that Paigen's diet feeding *per se* did not reduce the SR-B1 transcription in wild type mice (**Fig. 5**). However, in mice lacking apoE the atherogenic diet feeding markedly diminished its transcription. When apoE-lacking mice were fed a Paigen's diet and orally administrated with 10–20 mg/kg PPE or α-asarone, the transcription of hepatic SR-B1 was highly enhanced (**Fig. 5**). Therefore, treating with PPE and α-asarone may alleviate atheroma formation in the vessels through regulating HDL clearance.

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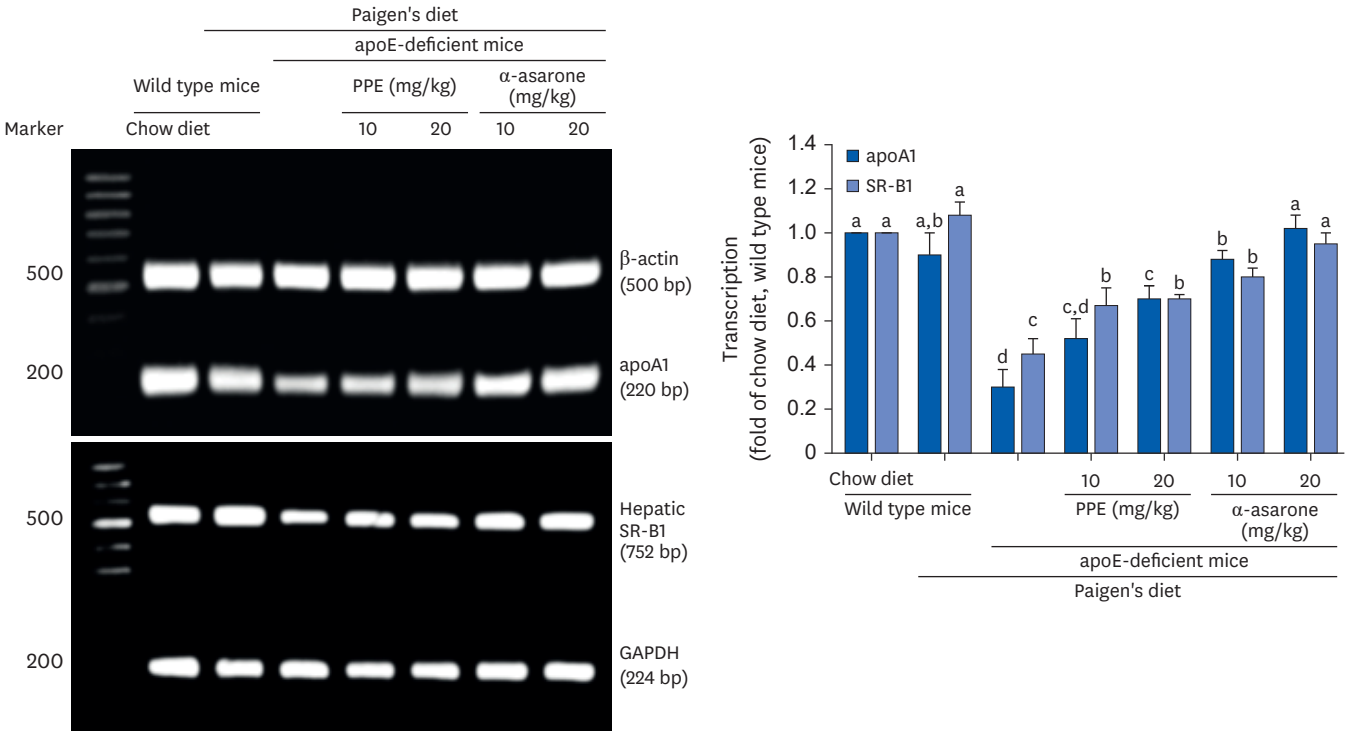

Fig. 5. Upregulation of hepatic transcription of apoA1 and SR-B1 by PPE and α-asarone. Wild type and homozygous apoE-knockout C57BL/6N mice (5 wk of age) were fed either chow diet or atherogenic Paigen's diet. Atherogenic Paigen's diet-fed apoE-deficient mice were divided into 5 subgroups. These apoE-deficient mice received 10–20 mg/kg PPE or 10–20 mg/kg α-asarone via gavage daily for 10 wk. For the measurements of the mRNA levels of hepatic apoA1 and SR-B1, the reverse transcription polymerase chain reaction analysis was conducted with mouse primers of apoA1 and SR-B1. β-Actin and GAPDH were used as internal controls. Values in bar graphs (n = 4 separate experiments) not sharing the same lower case alphabet letter indicate a significant difference at $P < 0.05$.
Apo, apolipoprotein; SR-B1, scavenger receptor B1; PPE, purple perilla frutescens extract; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION

The atheroprotective apoE interacts with the LDLR and SR-B1 to engage in normal catabolism of TG-rich lipoproteins as well as cholesterol metabolism [8,9,28]. Accordingly, apoE deficiency or abnormality causes pathological disorders of CVD and neurological syndromes [10,29]. In the current study Paigen's diet highly enhanced plasma level of LDL cholesterol with a substantial increase in plasma TG level even in wild type mice. After this atherogenic diet, mice lacking apoE clearly exhibited the atherosclerotic phenotype with hypecholesterolemia and aortic atheroma. These finding indicate that apoE-deficient mice can malfunction the hepatic clearance of plasma lipoproteins and promote the foam cell formation of peripheral tissue macrophages. In fact, the transcription of hepatic SR-B1 was attenuated in mice lacking apoE receiving the Paigen's diet, indicating uptake of cholesterol carried by HDL from peripheral tissue was suppressed. The plasma levels of CETP and PLTP was significantly elevated in mice with hypercholesterolemia, but the LCAT level was minimally affected. Lack of CETP and PLTP is known to increase HDL and lower LDL levels [30]. However, the role of LCAT in atherosclerosis is still unclear [31]. Inhibition of CETP and PLTP can be a new approach to controlling atherogenic CVD. In addition, blood monocyte activation and inflammatory responses were observed in apoE-deficient mice fed on Paigen's diet. Induction of aortic CD68 and F4/80 increased in apoE-deficient mice fed on Paigen's diet, implying that atherogenic diet promoted the macrophage accumulation in aortic vessels.

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In the light of current knowledge, it may be reasonable to explore natural compounds that can diminish the incidence of atherosclerosis by reducing the abnormality of apoE-responsive cholesterol metabolism. There are several reports about the favorable effects of natural compounds on the pathological development due to apoE deficiency in the cardiovascular system [32-34]. The polyphenol chlorogenic acid promotes cholesterol efflux, reducing atherosclerosis development in apoE-knockout mice fed with a cholesterol-rich diet [34]. The current study revealed that PPE containing α-asarone inhibited hypercholesterolemia in atherogenic apoE-deficient mice with a marked decrease in plasma LDL-C. It should be noted that α-asarone greatly lowered the AI of apoE-knockout mice with increased HDL-C. Our previous studies have shown that PPE with α-asarone enhanced cholesterol efflux from oxidized LDL-exposed macrophages [17], In addition, α-asarone blocks 7β-hydroxycholesterol-exposed macrophage injury through promoting autophagy and diminishing ER stress [35,36]. This study showed that PPE and α-asarone blocked the atheroma formation in the aortas of atherogenic apoE-lacking mice. Therefore, the inhibition of aortic atheroma by PPE and α-asarone may entail a slight increase in cholesterol efflux to HDL in macrophages.

Several studies have shown potential mechanisms responsible for the atheroprotective properties of natural compounds in apoE-knockout mice [37-39]. Anthocyanin-rich black elderberry extract improves inflammation-related impairments in HDL function and reduces aortic cholesterol in hyperlipidemic apoE-deficient mice by impacting hepatic gene expression [37]. The current study showed that PPE and α-asarone lowered plasma levels of MCP-1 and IL-1β, alleviating systemic inflammation of apoE-deficient mice fed on atherogenic diet. The PPE containing α-asarone enhanced hepatic transcription of apoA1 and SR-B1, indicating hepatic HDL-C uptake and normal HDL function. In addition, PPE and α-asarone reduced the aortic macrophage infiltration in parallel with a significant decrease in fat accumulation in the aorta of Paigen diet-fed mice. These findings suggest that PPE with α-asarone may encumber inflammation-responsive atheroma formation in atherosclerotic mice. Cannabis seed oil ameliorates experimental atherosclerosis through regulating high cholesterol diet-induced vascular inflammation in apoE-knockout mice [40]. On the other hand, PPE and α-asarone controlled plasma levels of lipid transfer proteins in apoE-deficient mice with diet-induced hyperlipidemia. One investigation shows that polydatin, a natural precursor of resveratrol, suppresses high fat diet-induced atherosclerosis in apoE-deficient mice through enhancing RCT [41]. PPE and α-asarone inhibited the CETP induction in cholesterol diet-fed apoE-knockout mice, carrying a larger cholesterol load to the liver for cholesterol secretion without directing hepatic uptake of LDL-C and VLDL-C by LDL receptors. Much effort has been made to develop HDL- and RCT-targeted drug therapies, but more investigation is required on how naturally-occurring dietary compounds affect HDL function and atherosclerosis [42].

In summary, the current study demonstrated that PPE containing α-asarone reduced inflammation-responsive atheroma formation in diet-induced atherogenic apoE-deficient mice (**Fig. 6**). Cholesterol-rich diet enhanced systemic inflammation and aortic macrophage infiltration suppressed by PPE and α-asarone. In addition, PPE and α-asarone inhibited the CETP induction of and conversely enhanced hepatic transcription of apoA1 and SR-B1, hence promoting hepatic uptake of HDL-C for cholesterol secretion. Accordingly, α-asarone in PPE interfered with diet-induced atherosclerosis, and restored HDL dysfunction in the absence of apoE. Although PPE can act as a regulator of CETP and LCAT against inflammation in rodents, the clinical role of PPE is still unclear. In addition, further work on safety is needed.

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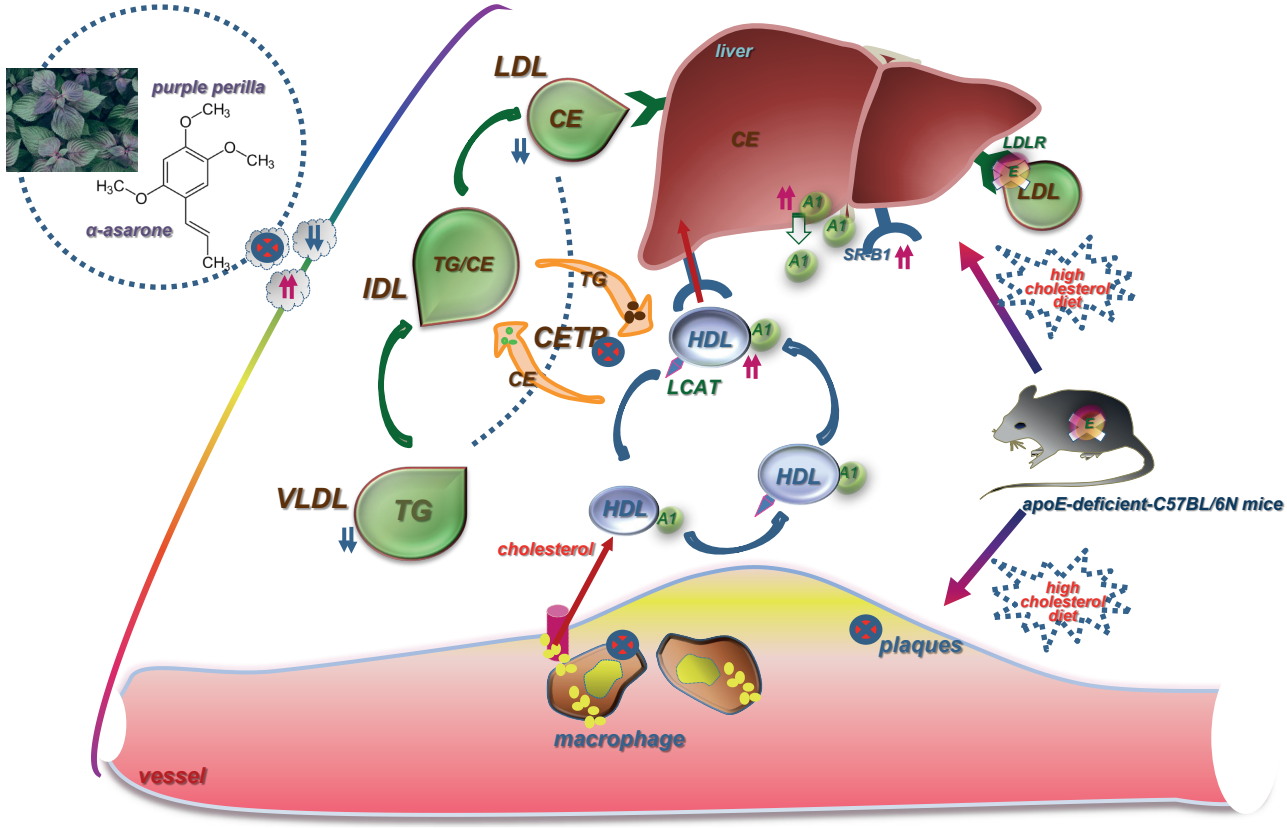

Fig. 6. Schematic diagram showing the effects of PPE and α-asarone on atherosclerosis in apoE-deficient mice. PPE and α-asarone can alleviate atherosclerosis by reducing inflammation and improving liver HDL clearance in apoE-deficient mice vulnerable to atherosclerosis.
LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; CE, cholesterol ester; TG, triglycerides; PPE, purple perilla frutescens extract; apoE, apolipoprotein E.

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