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Effect of *Siegesbeckia glabrescens* Extract on Foam Cell Formation in THP-1 Macrophages

Ha-Rin Moon and Jung-Mi Yun

Department of Food and Nutrition, Chonam National University, Gwangju 61186, Korea

ABSTRACT: The accumulation of cholesterol-bearing macrophage foam cells in the initial stages of atherosclerosis serves as a characteristic feature of atherosclerotic lesions. The inhibitory effect of *Siegesbeckia glabrescens*, a species of flowering plant in the Asteraceae family, on foam cell formation in THP-1 macrophages has not yet been elucidated. In this study, we explored the effect of *S. glabrescens* ethanol extract (SGEE) and hot water extract (SGWE) on foam cell formation via co-treatment with oxidized low density lipoprotein (ox-LDL) and lipopolysaccharide (LPS), mimicking the occurrence of atherosclerosis *in vitro*, and studied the regulation of its underlying mechanisms. THP-1 cells differentiated by PMA (1 μM) for 48 h were subsequently treated with/without SGWE and SGEE for 48 h. THP-1 macrophages were treated with ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h. Treatment with ox-LDL and LPS for 24 h enhanced the lipid accumulation in foam cells compared to in untreated cells, as determined by oil red O staining. In contrast, SGWE and SGEE treatment inhibited lipid accumulation in foam cells. Both extracts significantly upregulated ABCA1, LXRα, and PPARγ expression in ox-LDL- and LPS-treated cells ($P<0.05$). Moreover, both SGWE and SGEE decreased LOX-1, CD36, and SR-A1 expression. The co-treatment of ox-LDL and LPS increased NF-κB, COX-2, and pro-inflammatory activation and expression compared with untreated cells. However, this increase suppressed NF-κB, COX-2, and pro-inflammatory expression by SGWE and SGEE. The results indicated that both extracts can partially inhibit foam cell formation and contribute to protective effects by suppressing cholesterol accumulation during the onset of atherosclerosis.

Keywords: atherosclerosis, foam cells, lipopolysaccharides, oxidized low density lipoprotein, *Siegesbeckia glabrescens*

INTRODUCTION

Atherosclerosis is a chronic progressive inflammatory disease in which cholesterol accumulation and chronic inflammation affect the blood vessels, resulting in the progress of atherosclerotic plaques (Ruparelia et al., 2017; Libby et al., 2019; Orekhov et al., 2020). Due to hyperlipidemia, a major risk factor for atherosclerosis, monocytes migrate into the blood vessel wall and differentiate into macrophages (Escate et al., 2016). Blood low density lipoprotein (LDL) is accumulated in vascular endothelial cells and then converted to oxidized low density lipoprotein (ox-LDL) by macrophages. Foam cells are then generated through the phagocytosis of macrophages and accumulate on the inner wall cell (Yan et al., 2020). From the development of initial atherosclerosis lesions to the progression of complex plaque formation, macrophage-derived foam cells play important roles in atherosclerosis (Javadifar et al., 2021).

Foam cell formation occurs due to an imbalance between lipid accumulation and cholesterol efflux (Voloshyna et al., 2013). Ox-LDL, absorbed by scavenger receptors (SRs), accumulates lipids and causes cholesterol accumulation in foam cells, contributing to atherogenesis (Itabe et al., 2011). The expression of several SRs, such as SR class A1 (SR-A1), cluster of differentiation 36 (CD36), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), causes the internalization of ox-LDL, resulting in lipid accumulation and foam cell transformation (Ouyang et al., 2023). In addition, foam cells generated by the absorption of ox-LDL via SRs stimulate inflammatory responses and accelerate blood vessel walls thickening (Malekmohammad et al., 2021).

Cholesterol efflux suppresses the transformation of macrophages into foam cells by reducing cholesterol ester accumulation in macrophages (Groenen et al., 2021). It has been demonstrated in cell, animal, and clinical studies that cholesterol efflux plays an important role in preventing atherosclerosis (Nakaya et al., 2010; Khera et al., 2011; Qian et al., 2021). ATP binding cassette sub-

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Correspondence to Jung-Mi Yun, E-mail: sosung75@jnu.ac.kr

family A member 1 (ABCA1), a membrane protein that plays a significant role in the cellular cholesterol efflux mechanism, mediates cholesterol efflux from foam cells (Kawashima and Medh, 2014). When peroxisome proliferator-activated receptor gamma (PPARγ) is activated in macrophages, liver-X-receptor α (LXRα) is expressed, and finally, ABCA1 expression is increased, resulting in increased cholesterol efflux (Ren et al., 2019). Therefore, the inhibition of lipid accumulation and foam cell formation is important to protect cells from atherosclerosis.

During the various stages of atherosclerosis, the differentiation into monocyte-derived foam cells promotes the aggregation of monocytes, leading to the release of many pro-inflammatory cytokines (Kapellos et al., 2019). Nuclear factor-kappa B (NF-κB), an important transcription factor in the inflammatory response, is activated by ox-LDL and affects early atherosclerosis, cholesterol efflux, and lipid accumulation (Liu et al., 2023). Accordingly, NF-κB is highly expressed in patients with atherosclerosis (Matsumori, 2023).

Although there are drugs that can significantly ameliorate the symptoms of cardiovascular diseases, they are known to effect liver and kidney function (Behlke et al., 2020). Food and natural materials are being increasingly explored as raw materials for the purpose of developing alternative drugs. *S. glabrescens*, a flowing plant in the family Asteraceae, grows naturally in Korea, Japan, Taiwan, and China and is a plant commonly grown along roadsides in Korea (Tao et al., 2018). Various biological activities, including antithrombotic, antioxidant, and antitumor activities have been reported for *S. glabrescens* extract (Cho et al., 2013; Jeon et al., 2014). However, the fundamental mechanisms of *S. glabrescens* extract regarding its effects on atherosclerosis are not yet completely understood. Therefore, in this study, we explored the mechanisms by which *S. glabrescens* extract affects cholesterol efflux, lipid accumulation, and inflammation-related gene expression in human monocyte THP-1 cell-derived macrophages.

MATERIALS AND METHODS

Reagents

S. glabrescens was purchased from an online open market (Gmarket). LPS, phorbol 12-myristate 13-acetate (PMA), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Human THP-1 cells were procured from the Korean Cell Line Bank. The BCA protein assay kit and ox-LDL were procured from Thermo Fisher Scientific. Unless otherwise stated, all other chemicals were procured from Sigma-Aldrich or Biosesang.

Preparation of *Siegesbeckia glabrescens* extract

S. glabrescens ethanol extract (SGEE) and hot water extract (SGWE) were prepared by adding 10 g of 80% ethanol (SGEE) or water (SGWE) per g of *S. glabrescens* powder, stirring for 1 h at 70°C under reflux cooling, and filtering twice. The obtained extract was concentrated under reduced pressure using a rotary vacuum evaporator (EYELA N-1000, Bio Konvision) and dried to calculate the solid content. The yields of SGWE and SGEE were calculated to be 24.18% and 18.11%, respectively. Both extracts were stored at −80°C until further use.

THP-1 cell culture and phorbol 12-myristate 13-acetate (PMA)-induced differentiation

Human THP-1 cells were cultured in RPMI 1640 medium (Welgene) supplemented with 10% fetal bovine serum (Welgene) and 1% antibiotics (Welgene) in an atmosphere of 5% CO_2 at 37°C. THP-1 cells were then treated with PMA (1 μM) for 48 h to differentiate them into macrophages. Differentiated THP-1 cells were cultured for 48 h in the presence or absence of SGWE (64 − 250 μg/mL) and SGEE (31 − 125 μg/mL) and then treated with ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h before being harvested. Subsequently, the culture medium was collected for cytokine secretion measurement, the cells were washed twice with phosphate-buffered saline (PBS, Biosesang), and the cells were harvested.

Measurement of cell viability

The cytotoxic effects of SGWE and SGEE on PMA-activated THP-1 macrophages were measured via MTT assay. The cells were seeded at a concentration of 1×10^6 cells/well in 24-well plates and treated with SGWE or SGEE for 48 h. The cells were then treated with ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h before the MTT assays. MTT solution (100 μL; 1 mg/mL) was added, and cells were incubated for a further 2 h. The precipitated formazan was solubilized in 1 mg/mL of 100% dimethyl sulfoxide. Finally, plates were placed in an EZRead 400 microplate reader (Biochrom) to measure the absorbance at 570 nm.

Oil red O (ORO) staining

Cells were examined for lipid inclusion via ORO staining. In brief, cells were incubated with 4% paraformaldehyde (PFA) for 30 min at 4°C and then treated with ORO solution (Sigma-Aldrich) for 30 min. Images were acquired using a Leica microscope (Leica Microsystems). Images were collected using the Leica Application Suite X software (Leica Microsystems). A 400× objective was used for all images. The degree of staining was quantified by measuring the absorbance at 520 nm using an EZRead 400 microplate reader (Biochrom).

Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected, and the cytokine levels were measured using ELISA IL-6 and TNF-α kits (Raybiotech) to assess the impact of SGWE and SGEE on cytokine production in PMA-activated THP-1 macrophages. Values were calculated based on a standard curve.

Immunoblotting analysis

Whole-cell lysates were prepared using RIPA buffer (Biosesang) supplemented with Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Nuclear lysates were prepared using a nuclear extraction buffer (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF) containing 10% NP-40. Lysate protein concentrations were measured using a BCA protein assay (Pierce) following the manufacturer's protocol. Proteins were separated via SDS-PAGE (20 μg), and the protein bands were transferred onto a nitrocellulose membrane (Invitrogen), reacted for 2 h in blocking buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk], and incubated with appropriate primary antibodies for 2 h. The SIRT1 was purchased from Cell Signaling Technology. NF-κB p65, LOX-1, PPARγ, LXRα, COX-2 and TNF-α were purchased from Santa Cruz Biotechnology. CD36, SR-A1, ABCA1 were purchased from Abcam. After incubation with the primary antibody and washing, the blot was then incubated with a diluted conjugated secondary antibody for 2 h. After applying the Western blotting luminol reagent (Santa Cruz Biotechnology) to the blot, the results were analyzed using the ChemiDoc™ XRS+ Imaging System (BioRad). Protein expression intensity was normalized to β-actin and quantified using ImageJ (free online image analysis software).

Quantitative real time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using Trizol™ reagent according to the manufacturer's protocol (Thermo Fisher Scientific). Total RNA concentration and purity were assessed by measuring the absorbance at 260 and 280 nm using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). An Omniscript® RT kit (QIAGEN) was used to synthesize first-strand cDNA from 1 μg of total RNA. SYBR® Green-based qPCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Biorad). All reactions were run in triplicate. Significance was determined from β-actin-normalized $2^{-\Delta\Delta CT}$ value comparisons.

Immunofluorescence staining

After SGWE and SGEE treatment, cells were washed twice in PBS, fixed with 4% PFA for 30 min at 4°C, and stained overnight with NF-κB antibodies (1:100 dilution, Santa Cruz Biotechnology). After air-drying, the slides were incubated with the secondary antibody (1:2,000 dilution, Invitrogen) for 60 min, then stained with DAPI (100 ng/mL, Beyotime) at 37°C to visualize the nuclei, and the samples were washed three times in PBS. Slides were washed twice in PBS, air-dried, treated with a mounting medium, and examined at 400× magnification under a fluorescence microscope. Images were collected using the Leica Application Suite X software (Leica Microsystems).

Statistical analysis

All experiments were repeated at least three times, and the data from each experiment were represented as the mean±SD. Significant differences among groups were determined by one-way ANOVA, followed by the Duncan multiple range test using SPSS version 25.0 (IBM Corp.). The specific significance values are specified in the figure legends, and statistical significance was defined as $P<0.05$.

RESULTS

S. glabrescens hot water extract (SGWE) and ethanol extract (SGEE) inhibited inflammation in lipopolysaccharide (LPS)-induced THP-1 cells

The cytotoxicity of SGWE and SGEE in the inflammatory environment caused by LPS treatment was measured using the MTT assay. As a result, no cytotoxicity was observed for SGWE and SGEE in both the LPS-treated and untreated groups (Fig. 1A and 1B). Thus, the nontoxic concentration ranges for SGWE (64–250 μg/mL) and SGEE (31–125 μg/mL) were used in the subsequent experiments. Western blotting was performed to determine whether the LPS-induced NF-κB and sirtuin 1 (SIRT1) expression in the nucleus was inhibited by SGWE and/or SGEE treatment. As shown in Fig. 1C and 1D, the expression of NF-κB and SIRT1 increased and decreased, respectively, in the inflammatory environment induced by LPS treatment. However, treatment with SGWE or SGEE decreased the expression of NF-κB and increased the expression of SIRT1.

Effects of SGWE and SGEE on foam cell formation

We evaluated the ability of SGWE and SGEE to suppress lipid accumulation and foam cell formation in THP-1 macrophages cotreated with ox-LDL and LPS via ORO staining. The extent of red staining indicates the degree of lipid accumulation. As shown Fig. 1E and 1F, strong red staining was observed in the macrophages cotreated with ox-LDL and LPS. However, lipid accumulation significantly declined in macrophages exposed to SGWE (64–250 μg/mL) or SGEE (31–125 μg/mL) ($P<0.05$).

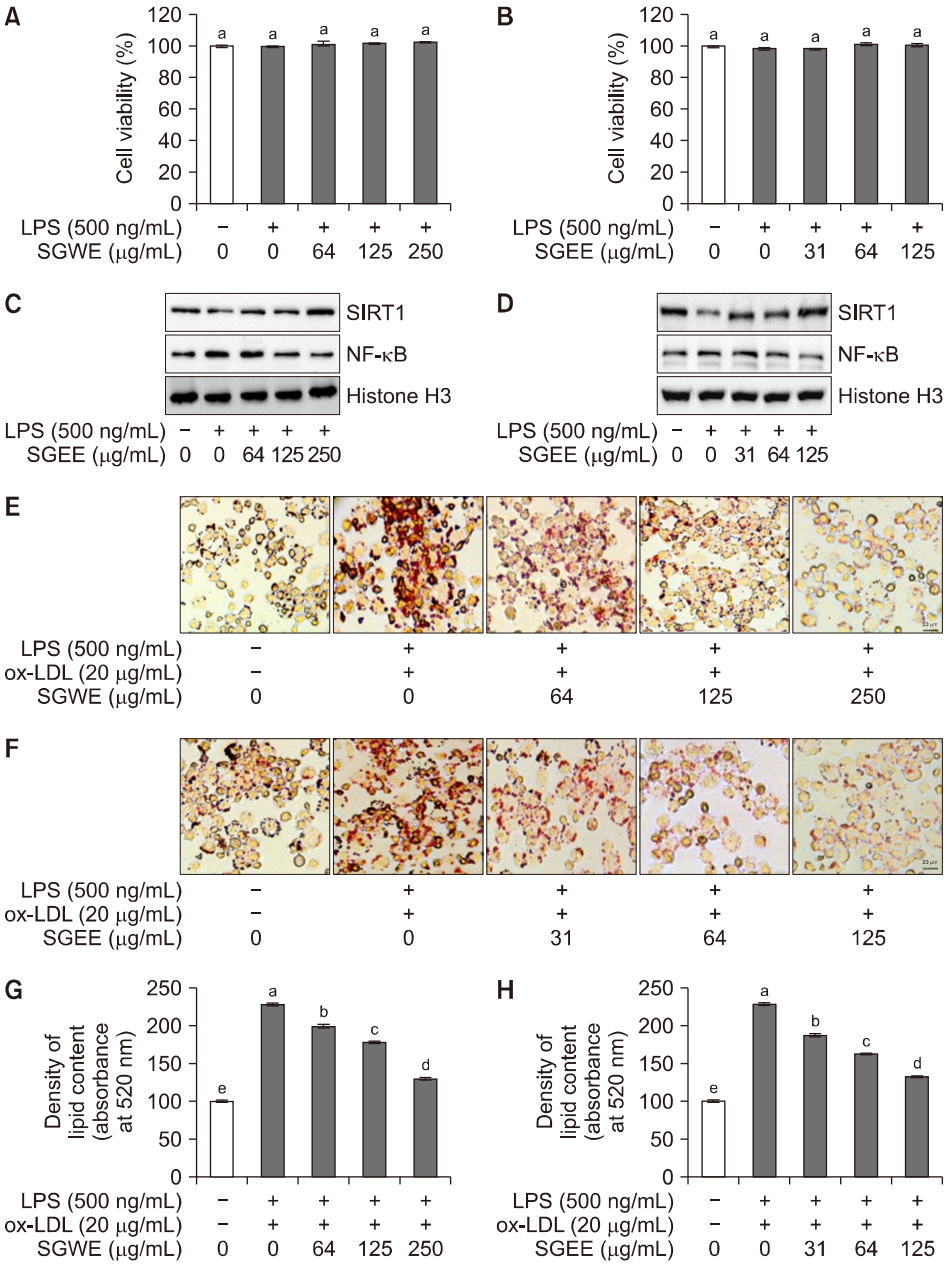

Fig. 1. Effect of LPS (500 ng/mL) on cell viability of THP-1 cells. LPS upregulated the expression of inflammatory factors, while SGWE/SGEE treatment downregulated lipid accumulation in THP-1 foam cells. (A and B) THP-1 monocytes were exposed to 1 μM of PMA for 48 h, pretreated with various concentrations of SGWE or SGEE, and then induced with or without 500 ng/mL LPS for 24 h. Cell viability was measured using the MTT assay. Experiments were performed in triplicate, and the results are presented as the mean±standard deviation. Different letters indicate significant differences (P<0.05), as determined by Duncan's multiple range test. The protein expression levels of (C) NF-κB and (D) SIRT1 were determined via immunoblotting. (E and F) Cells were stained with oil red O, and microphotographs were obtained using an optical microscope (magnification 400×). (G and H) Stained cells were dissolved in isopropanol solution, and the staining intensity was measured at 520 nm. LPS, lipopolysaccharides; PMA, phorbol 12-myristate 13-acetate; SGWE, *Siegesbeckia glabrescens* water extract; SGEE, *S. glabrescens* ethanol extract; ox-LDL, oxidized low density lipoprotein; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NF-κB, nuclear factor-kappa B; SIRT1, sirtuin 1.

These results showed that SGWE and SGEE suppressed the lipoprotein accumulation-inducing effect of ox-LDL and LPS and foam cell formation in THP-1 macrophages.

Effect of SGWE and SGEE on lipid receptor expression in foam cells

To investigate whether SGWE or SGEE treatment reduced lipid accumulation in macrophages cotreated with ox-LDL and LPS, we determined the expression degrees of CD36, SR-A1, and LOX-1 using immunoblotting and qRT-PCR. Co-treatment with ox-LDL and LPS significantly increased the expressions of CD36, SR-A1, and LOX-1. However, treatment with SGWE or SGEE resulted in reduced expression of CD36, SR-A1, and LOX-1 in THP-1 macrophages (P<0.05; Fig. 2A and 2B). Moreover, co-treatment with ox-LDL and LPS significantly upreg-

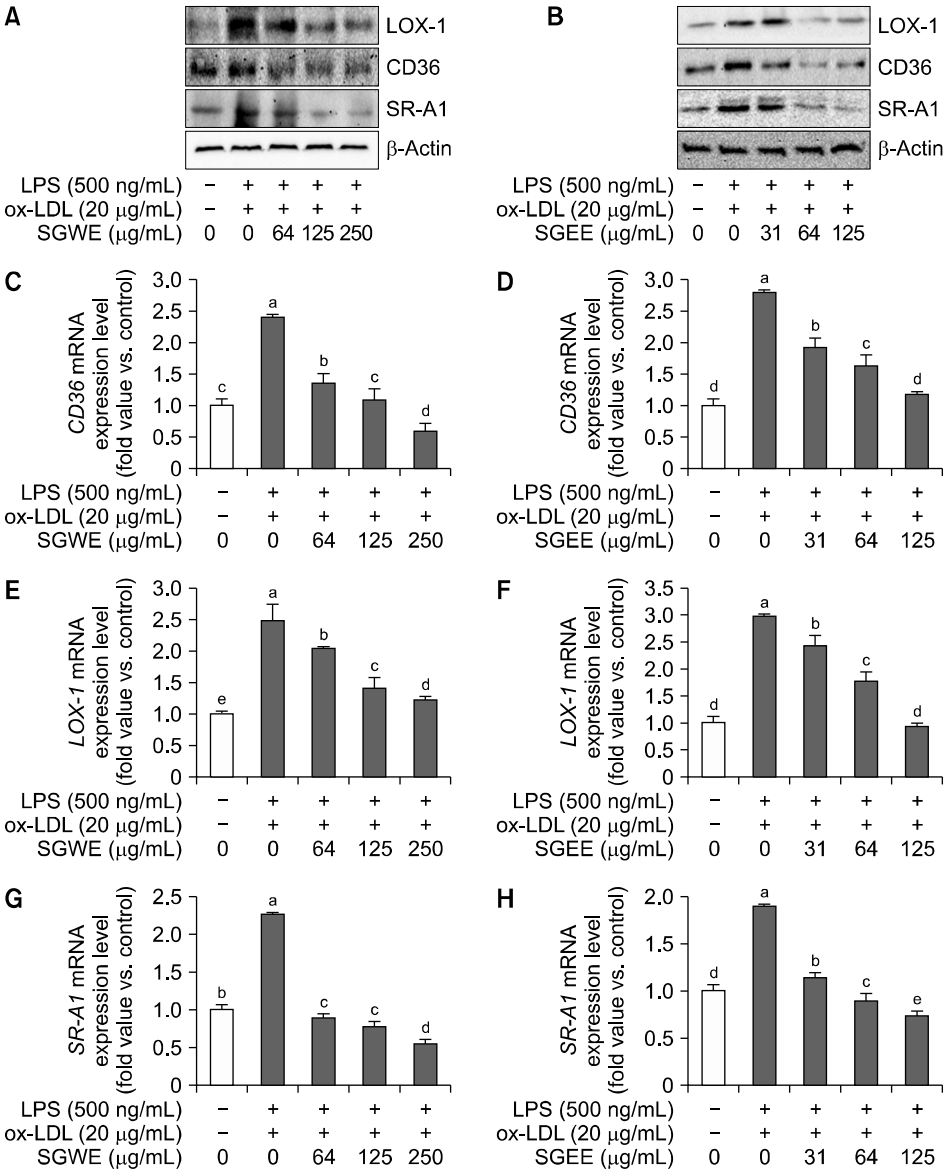

Fig. 2. Inhibition of SR-A1, CD36, and LOX-1 expression by SGWE and SGEE treatment in THP-1 foam cells. (A and B) The protein expression levels of SR-A1, CD36, and LOX-1 were determined using immunoblotting. (C–H) The relative mRNA expression levels of *CD36* (C and D), *LOX-1* (E and F), and *SR-A1* (G and H) were measured after normalization against *β-actin* mRNA expression. The data are expressed relative to the mRNA levels found in untreated cells, which were arbitrarily defined as one. Experiments were performed at least in triplicate, and the results are presented as the mean±standard deviation. Data were analyzed by applying the $2^{-\Delta\Delta CT}$ method. Different letters indicate significant differences (P<0.05), as determined by Duncan's multiple range test. SR-A1, scavenger receptor class A1; CD36, cluster of differentiation 36; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; SGWE, *Siegesbeckia glabrescens* water extract; SGEE, *S. glabrescens* ethanol extract; LPS, lipopolysaccharides; ox-LDL, oxidized low density lipoprotein.

ulated the mRNA levels of *CD36*, *SR-A1*, and *LOX-1* (P< 0.05). However, treatment with SGWE significantly decreased these levels 3-fold, 2.39-fold, and 3.03-fold, respectively, and treatment with SGEE decreased these levels 5.2-fold, 3.9-fold, and 3.2-fold, respectively, compared to the control (P<0.05; Fig. 2C–2H). These results suggest that SGWE and SGEE suppress foam cell formation by inhibiting lipid accumulation via the CD36/LOX-1/SR-A1 pathway.

Effect of SGWE and SGEE on cholesterol efflux in foam cells

We investigated the effects of SGWE and SGEE on cellular cholesterol efflux in foam cells using immunoblotting and qRT-PCR. As shown in Fig. 3A–3D, co-treatment with ox-LDL and LPS significantly decreased the expression of PPARγ, LXRα, and ABCA1 (P<0.05). However, SGWE and SGEE restored the levels of PPARγ, LXRα, and ABCA1. Moreover, co-treatment with ox-LDL and LPS significantly reduced the mRNA levels of *PPARγ*, *LXRα*, and *ABCA1* (P<0.05). However, treatment with SGWE significantly increased the mRNA levels of *PPARγ*,

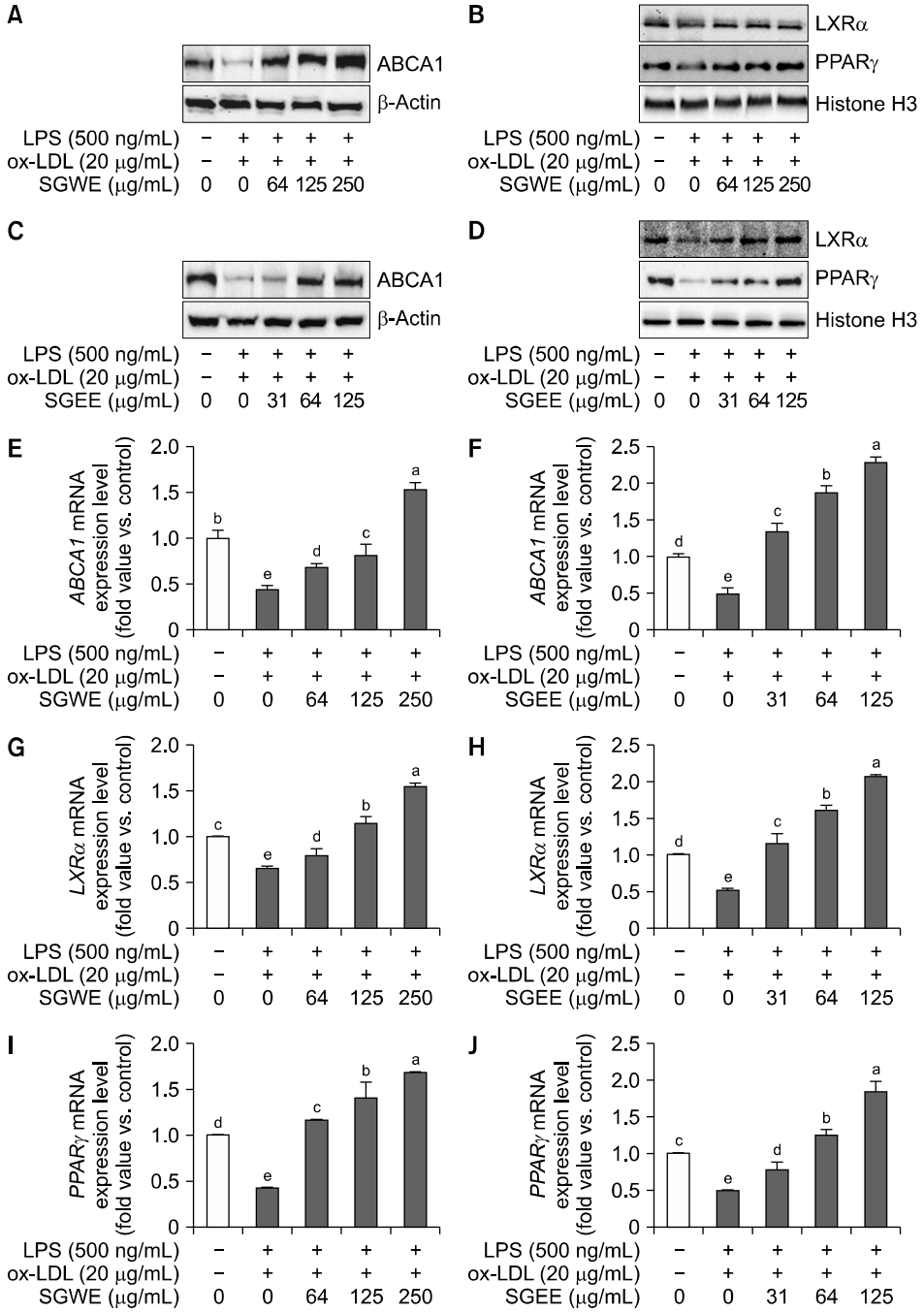

Fig. 3. Upregulation of ABCA1, LXRα, and PPARγ expression by SGWE or SGEE treatment in THP-1 foam cells. (A–D) The protein expression levels of ABCA1, LXRα, and PPARγ were determined using immunoblotting. Cells were harvested, and the expression of the mRNA levels of *ABCA1* (E and F), *LXRα* (G and H), and *PPARγ* (I and J) in ox-LDL- and LPS-induced foam cells were evaluated. Data are presented as the mean±standard deviation. Data were analyzed by applying the $2^{-\Delta\Delta CT}$ method. Different letters indicate significant differences ($P<0.05$), as determined by Duncan's multiple range test. ABCA1, ATP binding cassette transporter A1; LXRα, liver-X-receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; SGWE, *Siegesbeckia glabrescens* water extract; SGEE, *S. glabrescens* ethanol extract; ox-LDL, oxidized low density lipoprotein; LPS, lipopolysaccharides.

LXRα, and *ABCA1* 3.6-fold, 3-fold, and 5.3-fold, respectively, and treatment with SGEE increased these levels 1.8-fold, 2.1-fold, and 3.3-fold, respectively, compared to the control ($P<0.05$; Fig. 3E–3J). These results suggest that SGWE and SGEE can increase cholesterol efflux, thereby preventing foam cell formation induced by ox-LDL and LPS.

Effects of SGWE and SGEE on pro-inflammatory cytokine release and related gene expression via the nuclear factor-kappa B (NF-κB) pathway in foam cells

We examined pro-inflammatory cytokine secretion and NF-κB expression following SGWE or SGEE treatment in ox-LDL and LPS-induced foam cells. As shown in Fig. 4A–4D, the secretions of the inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-

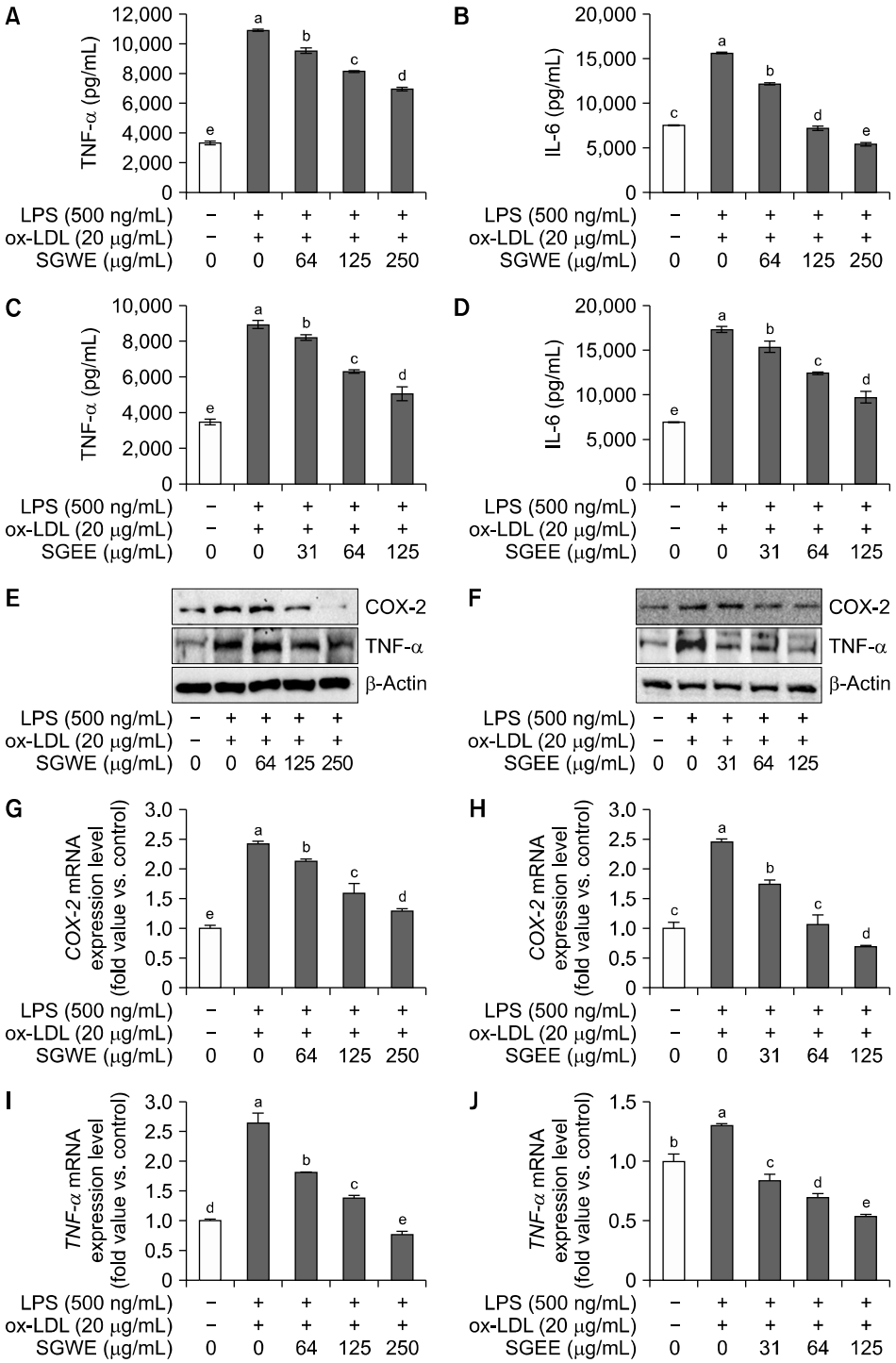

Fig. 4. Inhibition of inflammatory cytokine secretion by SGWE or SGEE treatment in ox-LDL- and LPS-induced foam cells. THP-1 foam cells were pretreated with different concentrations of SGWE (0–250 μM) for 48 h. (A–D) The secretion of IL-6 and TNF-α was measured using an ELISA kit. The expression levels of TNF-α and COX-2 were measured using (E and F) immunoblotting. Cells were harvested, and the expression of *COX-2* (G and H) and *TNF-α* (I and J) mRNA in ox-LDL- and LPS-induced foam cells was evaluated. Data are presented as the mean±standard deviation. Different letters indicate significant differences (P<0.05), as determined by Duncan's multiple range test. SGWE, *Siegesbeckia glabrescens* water extract; SGEE, *S. glabrescens* ethanol extract; ox-LDL, oxidized low density lipoprotein; LPS, lipopolysaccharides; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha; ELISA, enzyme-linked immunosorbent assay; COX-2, cyclooxygenase-2.

α) were significantly increased in ox-LDL- and LPS-induced foam cells, but this overproduction of cytokines was suppressed by SGWE treatment (by 82% and 77%, respectively) and by SGEE treatment (by 85% and 83%, respectively) (P<0.05). The protein expression results were similar to those obtained from the ELISA analysis (Fig. 4E–4J). Conversely, the expressions of COX-2 and TNF-α were attenuated by both SGWE and SGEE. NF-κB can accelerate the progression of atherosclerosis by increasing lipid uptake by macrophages and promoting

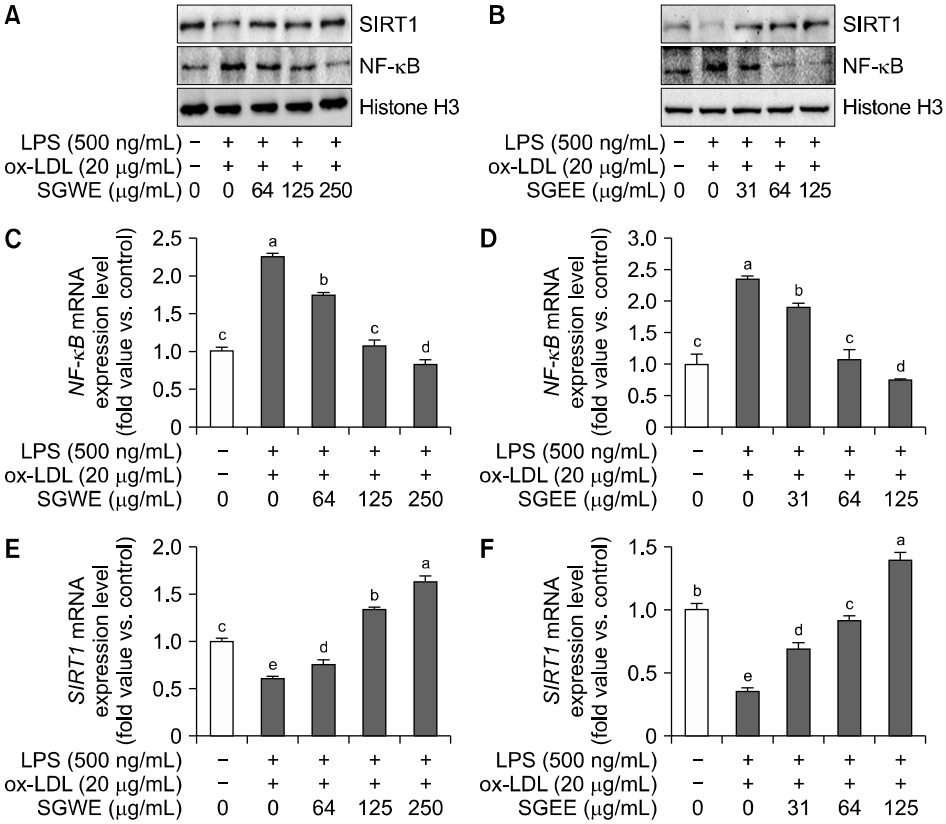

Fig. 5. Inhibition of NF-κB p65 activation by SGWE or SGEE treatment in ox-LDL- and LPS-induced foam cells. (A and B) The levels of NF-κB and SIRT1 proteins were measured using immunoblotting. Cells were harvested, and the expression of the mRNA level of *NF-κB* (C and D) and *SIRT1* (E and F) in ox-LDL- and LPS-induced foam cells was evaluated. Data are presented as the mean±standard deviation. Different letters indicate significant differences (P<0.05), as determined by Duncan's multiple range test. (G and H) THP-1 foam cells were treated with SGWE (0–250 μg/mL) and SGEE (0–125 μM) and fixed with 4% paraformaldehyde. After blocking with an appropriate buffer, cells were incubated with antibodies. Next, DAPI staining was performed to confirm the nuclei in the cells. Signals were quantified using fluorescence microscopy at 400× magnification. SGWE, *Siegesbeckia glabrescens* water extract; SGEE, *S. glabrescens* ethanol extract; ox-LDL, oxidized low density lipoprotein; LPS, lipopolysaccharides; NF-κB, nuclear factor-kappa B; SIRT1, sirtuin 1; DAPI, 4′,6-diamidino-2-phenylindole.

the transformation of macrophages into foam cells (Liu et al., 2023). As shown in Fig. 5A and 5B, SGWE and SGEE treatment significantly decreased NF-κB expression (P<0.05). SGWE and SGEE also significantly decreased the mRNA expression level of the *NF-κB* gene in foam cells compared to ox-LDL- and LPS-cotreated control cells (P<0.05; Fig. 5C–5F). Additionally, the nuclear translocation of p65 induced by ox-LDL and LPS was confirmed to be inhibited by treatment with 125 μg/mL of SGWE or SGEE through immunofluorescence analysis (Fig. 5G and 5H). These results indicate that SGWE and SGEE may serve as potential inhibitors of the inflammation caused by foam cells in the early stages of atherosclerosis.

DISCUSSION

Cardiovascular diseases are a leading cause of death and disability worldwide (Ha et al., 2015). According to the World Health Organization, in 2019, cardiovascular disease accounted for 32% of deaths worldwide, and the National Health and Nutrition Examination Survey reported that the prevalence of cardiovascular disease in Korea in the same year was 21.8% (WHO CVD Risk Chart Working Group, 2019; Oh et al., 2021). The mortality rates of cardiovascular diseases tend to rise sharply with age (Shin et al., 2020). Atherosclerosis, a major cause of cardiovascular disease, is a chronic inflammatory disease in which macrophages containing large quantities of lipids accumulate in the walls of blood vessels (Bobryshev et al., 2016). LDL in the arterial wall is converted to ox-LDL by certain risk factors, such as hyperlipidemia and alcohol consumption, and after ingesting ox-LDL, macrophages are converted to foam cells full of cholesterol (Gao et al., 2018; Higashi, 2023). Macrophage-derived foam cells play crucial roles in the onset and progression of atherosclerosis (Moore and Tabas, 2011); therefore, inhibiting the formation of macrophage-derived foam cells is an effective method for preventing and halting atherosclerosis.

Various natural dietary agents, such as epigallocatechin gallate, allicin, and cordycepin, have been explored for their ability to inhibit foam cell formation and cholester-

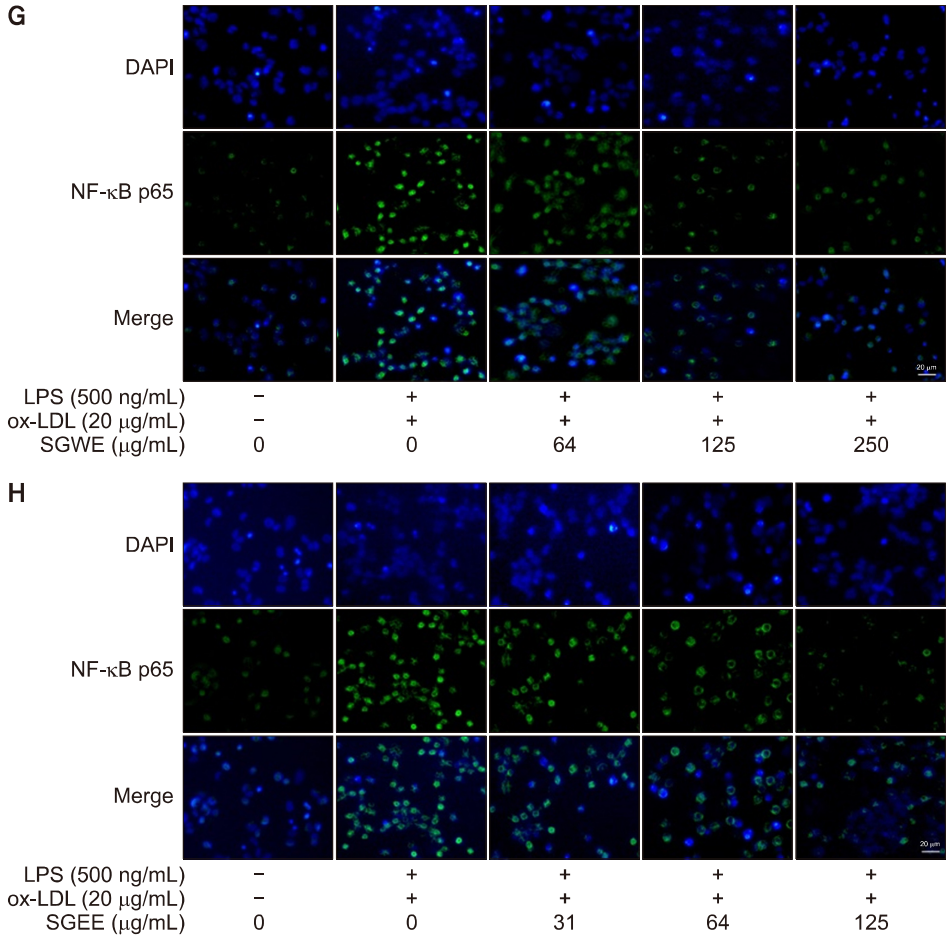

Fig. 5. Continued.

ol efflux (Yang et al., 2016; Li et al., 2017; Wang et al., 2019). However, it is necessary to improve the knowledge of biological mechanisms of bioactive molecules present in natural products in order to explain their effects. *S. glabrescens* contains kirenol, saponin, and tannin and is mainly used to treat high blood pressure and diabetes (Jiang et al., 2011; Zhong et al., 2019).

Atherosclerotic plaque formation begins with endothelial cell injury, which allows more LPS particles to penetrate the vascular wall. These lipoproteins, especially LDL, become trapped in the extracellular matrix of the intima. Subsequently, LDL undergoes modification and is absorbed by macrophages through specialized cell surface SRs, leading to the formation of foam cells. As lipids and foam cells continue to accumulate, the foam cells undergo apoptosis, creating a necrotic core composed of cell debris and cholesterol (Björkegren and Lusis, 2022).

There is a strong correlation between the concentrations of LDL and cholesterol in the serum, plaque formation, and the development of atherosclerosis (Podolecka et al., 2018). Accordingly, high concentrations of LDL and cholesterol in the serum are considered major risk factors for atherosclerosis. A previous study revealed that the main mechanism behind macrophage formation stems from disorders of ox-LDL intake and lipid efflux (Lu et al., 2022). Circulating monocytes, recruited to the vascular lamina after LDL uptake, differentiate into macrophages upon infiltration. When macrophages infiltrate the arterial wall, they absorb ox-LDL, forming foam cells, which are hallmarks of atherosclerosis (Lee and Choi, 2020). Ox-LDL is toxic to cells, and foam cells induce inflammatory responses and promote further recruitment of macrophages into the vessel, exacerbating inflammation and ultimately leading to plaque formation (Taleb, 2016). Therefore, mechanisms that inhibit foam cell formation in the early stages of atherosclerosis are expected to attenuate the progression of atherosclerosis.

Our results indicate that the combined treatment of ox-LDL and LPS promotes the accumulation of lipids within cells and increases the amount of ORO-stained lipid particles deposited intracellularly. Our data show that a concentration of 250 μg/mL of SGWE or 125 μg/mL of SGEE significantly decreased the formation of lipid droplets compared to that in untreated cells ($P< 0.05$). ORO staining revealed that SGWE and SGEE lowered foam cell formation and lipid uptake compared with ox-LDL- and LPS-cotreated THP-1 cells. Similar to this study, Park et al. (2015) reported that purple *Perilla frutescens* extract inhibited ox-LDL-induced foam cell formation in murine macrophages. Phang et al. (2020) re-

ported that maslinic acid inhibited the lipid accumulation induced by ox-LDL and suppressed foam cell formation in THP-1 macrophages.

Atherosclerosis is a chronic inflammatory disease. Numerous studies have confirmed that various cytokines play important roles in the progression of atherosclerosis and plaque instability (Levi et al., 2012; Fatkhullina et al., 2016). IL-6 is a cytokine that regulates the inflammatory response induced by leukocytes and other cells and is also considered to be a biomarker of inflammation (Tanaka et al., 2014). TNF-α is considered to be an effective pro-inflammatory mediator that promotes the expression of other inflammatory cytokines and adhesion molecules as well as increasing the apoptosis of vascular smooth muscle cells, thus promoting atherosclerosis and plaque instability (Liberale et al., 2021). NF-κB, a transcription factor of the inflammatory pathway, is an important factor that regulates gene expression during the early stages of atherosclerosis, which are characterized by cytokine and chemokine expression, atherogenesis, and vascular inflammatory responses (Liu et al., 2017). Abnormal activation of NF-κB has been detected in the blood of atherosclerosis patients (Matsumori, 2023). In addition, NF-κB activated by ox-LDL, which causes an inflammatory response in macrophages, induces the expression of pro-inflammatory cytokines, including IL-6 and TNF-α, thereby accelerating foam septa formation and atherosclerosis (Wang et al., 2015). Here, we confirmed that co-treatment with ox-LDL and LPS increased the protein expression of NF-κB, TNF-α, and COX-2 and decreased that of SIRT1. However, SGWE or SGEE treatment downregulated the expression of TNF-α, COX-2, and NF-κB and upregulated that of SIRT1. In particular, it was observed that the inflammatory cytokines TNF-α and IL-6 were significantly reduced by SGWE treatment (by 82% and 77%, respectively) and SGEE treatment (by 85% and 83%, respectively) ($P<0.05$). SGEE demonstrated a stronger inhibitory effect on inflammatory cytokines compared to SGWE. According to Zhang et al. (2017), berberine modulated LPS-induced inflammation in RAW 264.7 cells through SIRT1/NF-κB signaling. Li et al. (2018) conveyed that cinnamaldehyde attenuated nitric oxide, TNF-α, and IL-6 production through the NF-κB and mitogen-activated protein kinase pathway in ox-LDL-stimulated vascular smooth muscle cells. These findings suggest that SGWE and SGEE suppress LPS- and ox-LDL-induced inflammation by regulating NF-κB expression.

Managing cholesterol metabolism is important in the formation of foam cells. Recently, many studies using natural compounds have been conducted to control the cholesterol mechanism. The efflux of cholesterol from macrophages is crucial in preventing the formation of foam cells, which are hallmarks of the initial stages of atherosclerotic lesion development (Sun et al., 2015). ABCA1, a reverse cholesterol transport pathway, promotes the formation of high-density lipoprotein (HDL) particles in the peripheral stroma and mediates cholesterol efflux to lipid-poor apoA-1 (Phillips, 2018; Xu et al., 2022). In a previous study, the overexpression of ABCA1 in gene-modified mice was found to reduce the size of atherosclerotic lesions and increase HDL levels (Vaisman et al., 2012). In macrophages, ABCA1 has been shown to be a major target of LXRα in regulating cholesterol (Liang et al., 2015). LXRα plays an essential role in cholesterol release and the metabolism of very-low-density lipoproteins (Zhu et al., 2012). Furthermore, it acts as a transcription factor that regulates cholesterol homeostasis by stimulating the expression of ABCA1 transporters, thereby promoting cholesterol efflux (Chai et al., 2017). The expression of LXRα is regulated by PPARγ (Mandrekar-Colucci and Landreth, 2011), which is expressed in macrophages and not only suppresses the expression of inflammatory genes, including inducible nitric oxide synthase and TNF-α, but also plays a major role in maintaining lipid homeostasis in atherosclerosis as a key regulatory sensor of cell sterols and fatty acids (Wahli and Michalik, 2012; Gross et al., 2017). PPARγ has been shown to promote ABCA1-dependent cholesterol efflux by inducing the expression of LXRα in macrophages (Li et al., 2022a). This suggests that the PPARγ/LXRα signaling pathway can promote cholesterol clearance in macrophages by regulating ABCA1 expression (Liang et al., 2015). Rachmawati et al. (2023) reported that green tea extract suppresses foam cell atherosclerosis by reducing inflammation and improving the balance of cholesterol uptake and efflux through the upregulation of PPARγ/ABCA1. Chen et al. (2013) reported that *Hibiscus sabdariffa* leaf extract upregulated LXRα and ABCA1 in ox-LDL-induced murine macrophage J774A.1 cells.

Excessive influx of intracellular lipids is one of the main causes of foam cell formation (Poznyak et al., 2021). Lipid influx and efflux, regulated by SRs, play essential roles in the regulation of lipid homeostasis in macrophages. In macrophages, CD36, LOX-1, and SR-A1 internalize ox-LDL, leading to cholesterol accumulation and foam cell creation (Voloshyna et al., 2012; Li et al., 2018). Therefore, cholesterol metabolism regulation is intimately related to foam cell creation. CD36, an SR expressed on macrophages, has various functions, including fatty acid transport (Avraham-Davidi et al., 2013). It exhibits a higher affinity for ox-LDL compared to other SR family members, facilitating the internalization of ox-LDL into macrophages and promoting foam cell formation (Zhao et al., 2024). When CD36 recognizes ox-LDL, NF-κB expression increases, triggering a cascade of inflammatory responses that worsen the advancement of

atherosclerosis (Li et al., 2022b). It has also been reported that atherosclerotic lesions were reduced in CD36 knockout mice fed a high-cholesterol diet (Sheedy et al., 2013). LOX-1, a transmembrane glycoprotein that recognizes ox-LDL in endothelial cells, initiates plaque formation upon inducing ox-LDL endothelial dysfunction (Pirillo et al., 2013). Ox-LDL, through LOX-1, induces increased expression of leukocyte adhesion molecules on endothelial cells, activates cell death pathways, and enhances the generation of reactive oxygen species and expression of inflammatory genes (Kattoor et al., 2019). Recently, LOX-1 has been shown to be elevated in humans and animals with atherosclerosis (Jin and Cong, 2019). Yun et al. (2014) reported that kimchi methanol extract enhanced cholesterol efflux through the ABCA1/LXRα/CD36 signaling pathway. Zhuang et al. (2021) reported that amentoflavone inhibited lipid accumulation by suppressing CD36, SR-A1, and LOX-1 in ox-LDL-induced human artery smooth muscle cells.

In this study, we focused on the mechanisms of foam cell formation inhibition, specifically emphasizing the ABCA1/LXRα/PPARγ signaling pathway, associated with cholesterol efflux, and the CD36/LOX-1/SR-A1 signaling pathway, associated with lipid accumulation. Similar to previous research, our research revealed that SGWE and SGEE increases cholesterol efflux by inducing ABCA1/LXRα/PPARγ in THP-1 macrophages cotreated with ox-LDL and LPS and simultaneously inhibit lipid uptake by increasing the expression of SR-A1, CD36, and LOX-1. In particular, the cholesterol efflux genes ABCA1, LXRα, and PPARγ were significantly increased by 3.6-fold, 3-fold, and 5.3-fold, respectively, with SGWE treatment, and by 1.8-fold, 2.1-fold, and 3.3-fold, respectively, with SGEE treatment, compared to the control ($P<0.05$). In contrast, the lipid accumulation genes SR-A1, CD36, and LOX-1 were significantly reduced by 3-fold, 2.39-fold, and 3.03-fold, respectively, with SGWE treatment, and by 5.2-fold, 3.9-fold, and 3.2-fold, respectively, with SGEE treatment, compared to the control ($P<0.05$). Thus, SGEE demonstrated a stronger inhibitory effect on foam cell formation compared to SGWE.

The results of this study revealed that SGWE and SGEE inhibit the formation of foam cells induced by co-treatment of LPS and ox-LDL in THP-1-derived macrophages. SGWE and SGEE suppressed foam cell formation in conjunction with an increase in inflammatory cytokine production, as well as NF-κB and target gene expression in foam cells. Overall, these results demonstrate that SGWE and SGEE are effective natural substances for preventing and treating atherosclerosis. In the future, we intend to investigate the specific molecular mechanisms underlying lipoprotein accumulation and foam cell formation in ox-LDL- and LPS-cotreated THP-1 macrophages using active single compounds isolated from *S. glabrescens*.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: JMY. Analysis and interpretation: JMY, HRM. Data collection: JMY, HRM. Writing the article: HRM. Critical revision of the article: JMY. Final approval of the article: JMY. Statistical analysis: JMY. Obtained funding: JMY. Overall responsibility: JMY.

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