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p-Coumaric acid modulates cholesterol efflux and lipid accumulation and inflammation in foam cells

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Conflict of Interest
The authors declared no potential conflicts of interests.

ABSTRACT

BACKGROUND/OBJECTIVES: Atherosclerosis is a primary cause of cardiovascular disease associated with inflammation and lipid metabolism disorders. The accumulation of cholesterol-containing macrophage foam cells characterizes the early stages. The *p*-coumaric acid (*p*-CA) contained in vegetables may have various physiological activities. The inhibitory effect of *p*-CA on foam cell creation in THP-1 macrophages needs clarification. In this study, we explored the impact of *p*-CA on foam cells by co-treatment with oxidized low-density lipoprotein (ox-LDL) and lipopolysaccharides (LPS), mimicking the development of atherosclerosis *in vitro* and studied the regulation of its underlying mechanisms.
MATERIALS/METHODS: THP-1 cells differentiated by phorbol 12-myristate 13-acetate (1 μM) for 48 h and treated in the absence or presence of *p*-CA for 48 h. THP-1 macrophages were treated with combined ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays detected cell viability. Oil red O staining allowed us to observe lipid accumulation. Western blotting and quantitative polymerase chain reactions quantified corresponding proteins and mRNA.
RESULTS: Ox-LDL and LPS for 24 h enhanced the lipid accumulation using Oil red O in treated foam cells. By contrast, *p*-CA treatment inhibited lipid accumulation. *p*-CA significantly upregulated cholesterol efflux-related genes such as ATP binding cassette transporter A1, liver-X-receptor α and peroxisome proliferator-activated receptor gamma expression. Moreover, *p*-CA decreased lipid accumulation-related gene such as lectin-like oxidized low-density lipoprotein receptor-1, cluster of differentiation 36 and scavenger receptor class A1 expression. Combined ox-LDL and LPS increased nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and pro-inflammatory (tumor necrosis factor-α [TNF-α] and interleukin [IL]-6) activation and expression compared with untreated. *p*-CA suppressed this increased expression of NF-κB and COX-2, TNF-α and IL-6.
CONCLUSION: *p*-CA may play a vital role in atherosclerosis inhibition and protective effects by suppressing lipid accumulation and foam cell creation by increasing cholesterol efflux and can be potential agents for preventing atherosclerosis.

Keywords: Atherosclerosis; foam cells; inflammation; oxidized low density lipoprotein; *p*-coumaric acid

Author Contributions

Conceptualization: Yun JM; Funding acquisition: Yun JM; Investigation: Yun JM, Moon HR; Methodology: Yun JM, Moon HR; Supervision: Yun JM; Visualization: Moon HR; Writing - original draft: Moon HR; Writing - review & editing: Yun JM.

INTRODUCTION

The incidence of numerous cardiovascular diseases, including arteriosclerosis and heart disease, is increasing due to various factors such as westernized eating habits and lack of physical activity [1,2]. Atherosclerosis, one of the risk elements for cardiovascular disease, is a chronic inflammatory disease of arterial blood vessels characterized by oxidized lipid accumulation, foam cells, and inflammatory cytokines [3]. Foam cells, which play a vital role in the initiation phase of atherosclerosis, are differentiated from monocytes [4,5]. In the early stages of atherosclerosis, monocytes migrate to the arterial intima, and low-density lipoprotein (LDL) flows into the artery and is oxidized to oxidized low-density lipoprotein (ox-LDL) [6]. Monocytes differentiate into macrophages, absorb lipoproteins, and form foam cells [7].

Intracellular lipid homeostasis in macrophages dynamically regulates foam cell development by cholesterol efflux and ox-LDL uptake [8]. Lipid accumulation in macrophages through the scavenger receptor (SR) pathway induces endoplasmic reticulum stress, activates nuclear factor-κB (NF-κB) signaling, and stimulates the production of inflammatory cytokines, thereby transforming into lipid-laden foam cells [9,10]. In *in vitro* and *in vivo* studies, increased lipid accumulation through SRs accelerated atherosclerotic lesion formation [11,12]. Macrophages express SRs, such as scavenger receptor class A1 (SR-A1), cluster of differentiation 36 (CD36), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which absorb modified LDL and promote intracellular lipid accumulation, generating lipid-laden foam cells [13]. Additionally, SR in macrophages upregulates inflammatory cytokines, leading to cholesterol accumulation by macrophages. Macrophage cholesterol efflux can reduce cholesteryl esters and reduce atherosclerotic plaques [14]. Cell and animal studies have conveyed that cholesterol efflux plays a role in preventing atherosclerosis [15-17]. The clearance of macrophages carrying excessive influx of modified LDL from the arterial wall is mediated by cholesterol efflux, where intracellular lipids are transported to the liver by ATP binding cassette transporter A1 (ABCA1) and ATP binding cassette transporter G1 (ABCG1) [18]. ABCA1, which produces precursors of high-density lipoprotein (HDL) particles, plays a central role in maintaining cholesterol homeostasis and preventing atherosclerosis by promoting cholesterol and ox-LDL efflux [19]. Peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor-α (LXRα) contribute to the inhibition of foam cell creation by causing cholesterol to leak into the extracellular space and induce the transcription of ABCA1 and ABCG1 [20-22]. Activation of the PPARγ/LXRα/ABCA1 pathway may prevent atherosclerosis by enhancing cholesterol efflux.

Inflammation plays an essential role in forming foam cells in the development and progression of atherosclerosis [23]. Foam cells created by phagocytosing ox-LDL due to vascular endothelial cell damage appear to be involved in the creation and progression of atheromatous plaques by secreting cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, thereby inducing cell necrosis and thrombus formation [24,25]. In the inflammatory response, ox-LDL stimulates signaling in NF-κB and increases cholesterol accumulation, promoting the development of atherosclerosis [26]. NF-κB, one of the key regulators of inflammation, is an important transcription factor involved in plaque formation and progression of atherosclerosis [27]. Activation of NF-κB in macrophages accelerates atherosclerotic plaque formation by increasing the expression of inflammatory cytokines and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), IL-12, and TNF-α [28-30]. Several current studies have verified that sirtuin 1 (SIRT1) is a key regulator of atherosclerosis formation and progression [31]. In macrophages and endothelial cells, SIRT1 suppresses

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NF-κB transcriptional activity and reduces developmental responses by regulating pro-inflammatory cytokines including TNF-α and IL-6 [32,33]. A recent study found that high-fat diet-induced NF-κB and inflammatory cytokines were reduced in SIRT1-overexpressing mice, thereby reducing hepatic lipid accumulation [34]. Therefore, suppressing inflammation and improving macrophages oxidized lipid influx and efflux mechanisms are essential to preventing atherosclerosis.

Anti-atherosclerosis drugs lower cholesterol levels, while side effects such as gastrointestinal symptoms, muscle pain, and insomnia have been reported [35]. Recent research has increasingly focused on phytochemicals derived from safe, natural products such as ferulic acid, anthocyanin, and resveratrol, which have no known side effects [36-38]. Notably, laquinimod has been shown to prevent atherosclerosis by inhibiting monocyte adhesion to human aortic endothelial cells, primarily through the reduction of inflammatory gene expression, including IL-6 and MCP-1 [39]. Additionally, the administration of puerarin to apolipoprotein E knockout mice has demonstrated a protective effect against atherosclerosis by decreasing the proliferation of vascular smooth muscle cells (VSMCs) and reducing the expression of IL-8 [40]. The phenolic compound *p*-coumaric acid (*p*-CA) is found in fruits, vegetables, and plants such as cranberries and tomatoes [41,42]. *p*-CA has been reported to have various activities such as antioxidant, anti-dementia, and anti-angiogenesis [43-45]. Nevertheless, the study of atherosclerosis and its underlying mechanisms for *p*-CA is not elucidated. Therefore, this study explored how *p*-CA affects cholesterol efflux, lipid accumulation and inflammation-related gene expression in human monocyte THP-1 cell-derived macrophages.

MATERIALS AND METHODS

Materials

p-CA, lipopolysaccharides (LPS), phorbol 12-myristate 13-acetate (PMA), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human THP-1 cells were procured from the Korean Cell Line Bank (Seoul, Korea). The BCA protein assay kit and ox-LDL were procured from Thermo Fisher Scientific (Waltham, MA, USA). Unless otherwise stated, all other chemicals were procured from Sigma-Aldrich or Biosesang (Seongnam, Korea).

THP-1 cell culture and PMA-induced differentiation

Human THP-1 cells were cultured in RPMI 1640 (Welgene, Daegu, Korea) medium supplemented with 10% fetal bovine serum and 1% antibiotics (Welgene) in an atmosphere of 5% CO_2 at 37°C. THP-1 cells were treated with PMA (1 μM) for 48 h to induce differentiation into macrophages. THP-1 cells hatched in the presence or absence of numerous concentrations of *p*-CA (0–20 μM) were cultured for 48 h and then treated with ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h before harvest. Subsequently, the culture medium for cytokine secretion measurement was collected, the cells were washed twice with phosphate-buffered saline (PBS; Biosesang), and the cells were harvested.

Measurement of cell viability

The cytotoxic effects of *p*-CA on PMA-activated THP-1 macrophages were measured using the MTT assay. The cells were seeded at 1×10^6 cells/well in 24-well plates and treated with *p*-CA for 48 h. The cells were treated with ox-LDL (20 μg/mL) and LPS (500 ng/mL) for 24 h before

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the MTT assay. MTT solution (100 μL; 1 mg/mL) was added and incubated for a further 2 h. The precipitated formazan was solubilized in 1mg/mL of 100% dimethyl sulfoxide. Finally, plates were placed in a plate reader (EZRead 400 microplate reader; Biochrom, Cambridge, UK) to measure absorbance at 570 nm.

Oil red O staining

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

Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected, and cytokine levels were measured using IL-6 and TNF-α ELISA kits (Raybiotech, Norcross, GA, USA) to assess the impact of *p*-CA 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 was measured by a BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's protocol. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis separated proteins (20 μg), and protein bands were transferred onto a nitrocellulose membrane (Invitrogen, Waltham, MA, USA), reacted for 2 h in a 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. 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, Dallas, TX, USA) to the blot, the results were analyzed using the ChemiDoc XRS+ Imaging System (BioRad, Hercules, CA, USA). Protein expression intensity was normalized to β-actin and quantified using ImageJ (a free online image analysis software).

Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was isolated using a Trizol reagent per the manufacturer's protocol (Thermo Fisher Scientific). Total RNA concentration and purity were assessed by measuring 260 and 280 nm absorbance using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). An Omniscript RT kit (QIAGEN, Hilden, Germany) synthesized first-strand cDNA from 1 μg of total RNA. SYBR green-based quantitative PCR 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 *p*-CA treatment, cells were washed twice in PBS, fixed with 4% PFA for 30 min at 4°C, and stained overnight with the NF-κB antibody (1:100 dilution; Santa Cruz Biotechnology). After air drying, the slides were incubated with a secondary antibody (1:2,000 dilution; Invitrogen) for 60 min, then stained with DAPI (100 ng/mL; Beyotime, Shanghai, China) at 37°C to stain

the nuclei, and the samples were washed 3 times with PBS. Slides were washed twice in PBS, air-dried, treated with a mounting medium, and examined at 400× magnification under a fluorescence microscope. Leica Application Suite X software collected images.

Statistical analysis

All experiments were repeated at least 3 times, and the data from each experiment were represented as mean ± SD. Significant differences among groups were determined by one-way analysis of variance, followed by the Duncan multiple range test using SPSS version 25.0 (SPSS Institute, Chicago, IL, USA). The specific significance values are specified in the figure legend, and statistical significance was defined as $P < 0.05$.

RESULTS

p-CA inhibits inflammation in LPS-induced THP-1 cells

The cytotoxicity of *p*-CA in the inflammatory environment caused by LPS treatment was measured through MTT. As a result, no cytotoxicity was observed for *p*-CA in both the LPS-treated and untreated groups (**Fig. 1A**). Thus, the non-toxic concentration range of

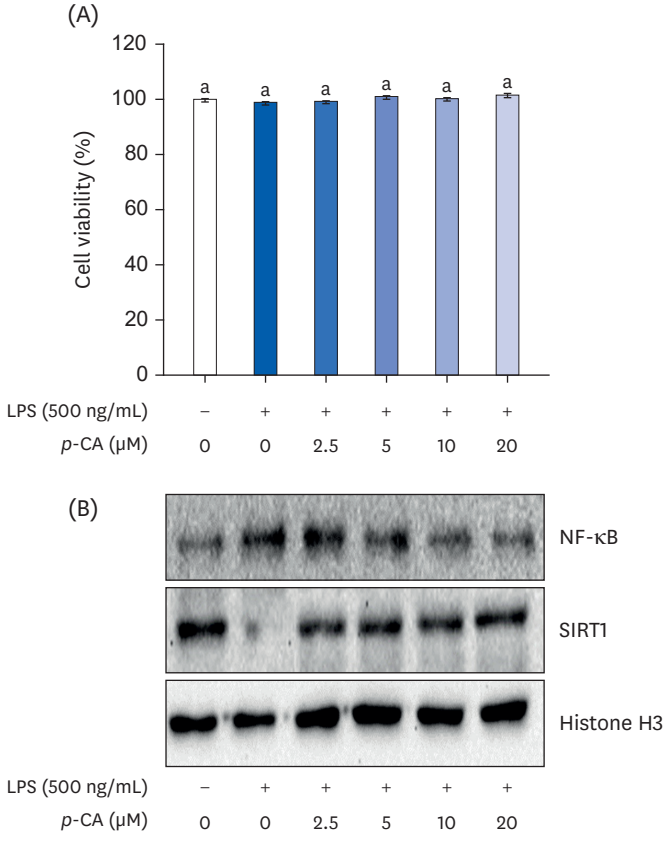

Fig. 1. Effect of *p*-CA on cell viability of LPS-treated THP-1 cells and upregulated expression of the inflammatory factor. (A) THP-1 monocytes were exposed to 1 μM of phorbol 12-myristate 13-acetate for 48 h, pretreated with *p*-CA at several concentrations and then induced with or without 500 ng/mL LPS for 24 h. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Experiments were performed in triplicate, and results are presented as the mean ± SD. The protein expression levels of NF-κB and SIRT1 were determined using immunoblotting (B).
LPS, lipopolysaccharides; *p*-CA, *p*-coumaric acid; NF-κB, nuclear factor-κB; SIRT1, sirtuin 1.
Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.

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p-CA (5–20 μM) was used in subsequent experiments. Whether *p*-CA inhibited LPS-induced NF-κB and SIRT1 expression in the nucleus was confirmed using western blotting. As shown in **Fig. 1B**, the expression of NF-κB increased, and the expression of SIRT1 decreased in the inflammatory environment induced by LPS treatment. However, treatment with *p*-CA reduced the expression of NF-κB and increased the expression of SIRT1.

Effects of *p*-CA on foam cell formation

Using Oil red O staining, we evaluated whether *p*-CA suppressed lipid accumulation and foam cell formation in ox-LDL and LPS co-treatment in THP-1 macrophages. **Fig. 2** shows strong red staining in macrophages after co-treatment ox-LDL and LPS. However, lipid accumulation significantly declined in macrophages exposed to *p*-CA (20 μM) ($P < 0.05$). These results showed that *p*-CA retarded the effect of ox-LDL and LPS on inducing lipoprotein accumulation and foam cell creation in THP-1 macrophages.

Effect of *p*-CA on lipid receptors expression in foam cells

To investigate whether there was reduced lipid accumulation by *p*-CA in ox-LDL and LPS co-treatment in THP-1 macrophages, we determined the expression degrees of CD36, SR-A1, and LOX-1 using immunoblotting and qPCR. Co-treatment with ox-LDL and LPS significantly increased the expression of CD36, SR-A1, and LOX-1. However, pretreatment with *p*-CA under combined ox-LDL and LPS-treated THP-1 macrophages resulted in significantly reduced

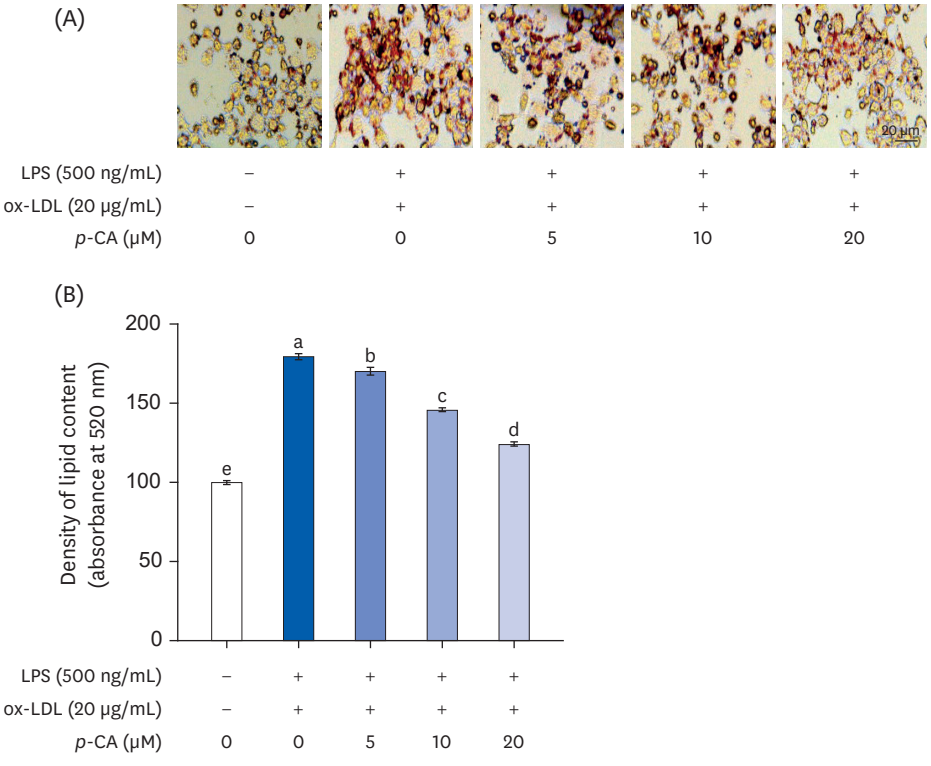

Fig. 2. Downregulation of lipid accumulation by *p*-CA treatment in THP-1 foam cells. THP-1 differentiated macrophages were cultured in the absence or presence of *p*-CA (0–20 μM) before 24 h. Then, the THP-1 cell was cultured in LPS (500 ng/mL) containing ox-LDL (20 μg/mL) for 24 h. (A) Cells were stained with Oil Red O; microphotographs were obtained using an optical microscope, magnification 400×. (B) Stained cells were dissolved in an isopropanol solution, and the staining intensity was measured at 520 nm. Experiments were performed in triplicate, and results are presented as the mean ± SD.
LPS, lipopolysaccharides; ox-LDL, oxidized low-density lipoprotein; *p*-CA, *p*-coumaric acid.
Different letters indicate significant differences ($P < 0.05$), as determined by Duncan's multiple range test.

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expression of CD36, SR-A1, and LOX-1 ($P < 0.05$) (**Fig. 3A**). Moreover, co-treatment with ox-LDL and LPS significantly upregulated the mRNA levels of *CD36*, *SR-A1* and *LOX-1*, but *p*-CA treatment significantly decreased the mRNA levels of *CD36*, *SR-A1* and *LOX-1* compared to the control ($P < 0.05$) (**Fig. 3B and C**). These results suggested that *p*-CA suppresses foam cell formation by inhibiting ox-LDL-and LPS-induced lipid accumulation.

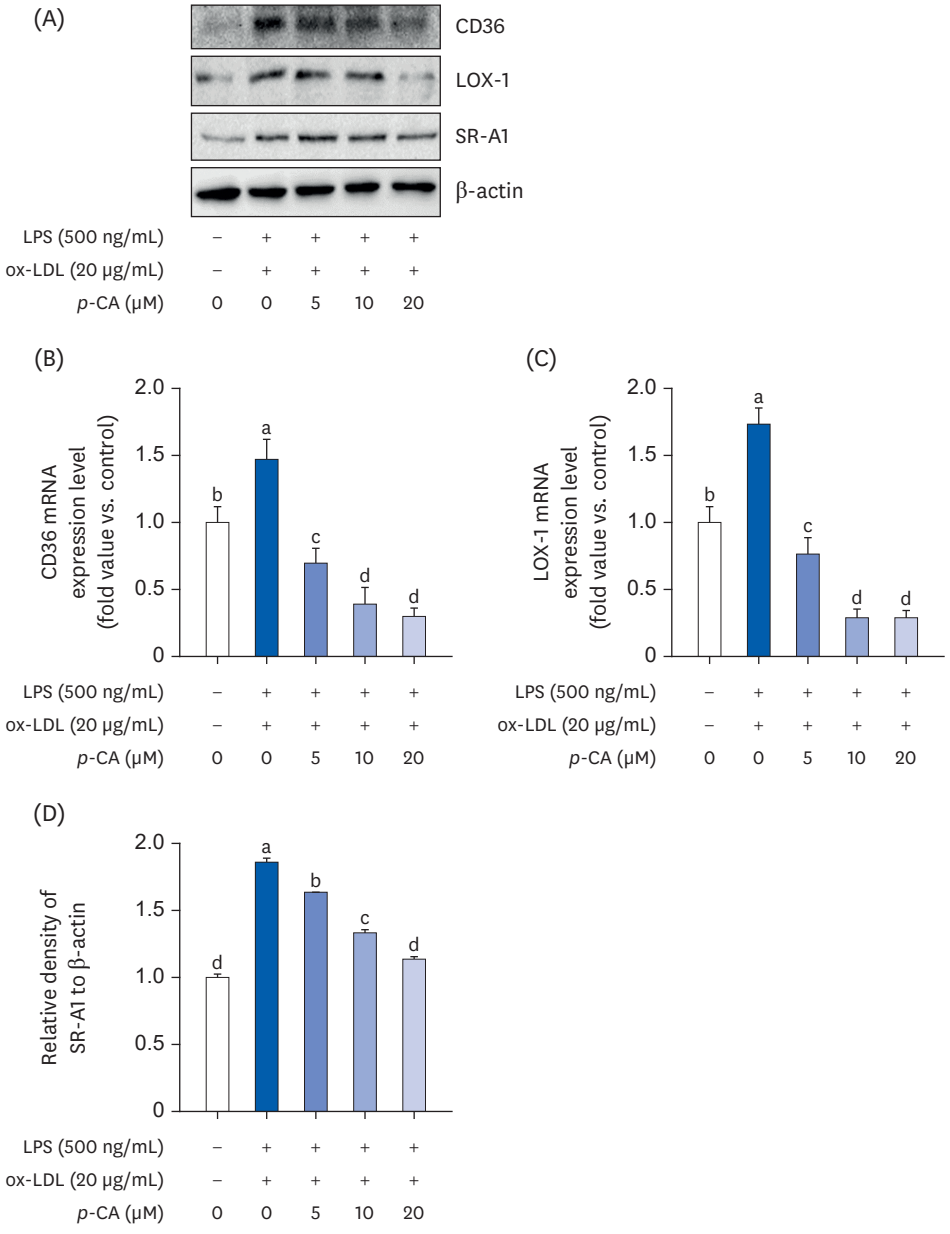

Fig. 3. Inhibition of SR-A1, CD36, and LOX-1 expression by *p*-CA treatment in THP-1 foam cells. The protein expression levels of SR-A1, CD36, and LOX-1 were determined using (A) immunoblotting. (B, C) The relative mRNA expression levels are shown after normalization against β-actin mRNA expression. (B) CD36 and (C) LOX-1 levels. The data are expressed relative to the mRNA levels found in untreated cells, which was arbitrarily defined as 1. (D) SR-A1 levels densities were normalized to β-actin using ImageJ software. Experiments were performed at least in triplicate, and the results are presented as mean ± SD. Data were analyzed by applying the $2^{-\Delta\Delta CT}$ method.
LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; SR-A1, scavenger receptor class A1; LPS, lipopolysaccharides; ox-LDL, oxidized low-density lipoprotein; *p*-CA, *p*-coumaric acid; CD36, cluster of differentiation 36.
Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.

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Effect of *p*-CA on cholesterol efflux in foam cells

We examined the effect of *p*-CA on cellular cholesterol efflux in foam cells using immunoblotting and qPCR. As shown in **Fig. 4A and C**, co-treatment ox-LDL and LPS decreased PPARγ, LXRα, and ABCA1 expression compared to the control ($P < 0.05$). However, *p*-CA reversed PPARγ, LXRα, and ABCA1. Moreover, the mRNA level of *PPARγ*, *LXRα*, and *ABCA1* was reduced in ox-LDL and LPS co-treatment compared to control, but *p*-CA treatment significantly increased the mRNA level of *PPARγ*, *LXRα*, and *ABCA1* compared to the control ($P < 0.05$) (**Fig. 4B, D and E**). These results suggested that *p*-CA could prevent ox-LDL-and LPS-induced foam cell formation by increasing cholesterol efflux.

Effects of *p*-CA on pro-inflammatory cytokine release and related gene expression via NF-κB pathway in foam cells

We examined inflammatory cytokines secretion and NF-κB expression by *p*-CA treatment in ox-LDL and LPS-induced foam cell formation. As shown in **Fig. 5A and B**, ELISA assays revealed that the inflammatory cytokines IL-6 and TNF-α secretion was significantly upregulated in ox-LDL and LPS-induced foam cell formation. Still, *p*-CA suppressed this overproduction of cytokines in ox-LDL and LPS-induced foam cell formation ($P < 0.05$). Protein expression results were similar to those obtained from the ELISA analysis (**Fig. 5C-E**). Conversely, cyclooxygenase-2 (COX-2) and TNF-α expression was downregulated by *p*-CA. In the early stages of atherosclerosis, NF-κB promotes the inflammatory response and facilitates the process of macrophages converting into foam cells by ingesting lipids [46]. As shown in **Fig. 6A**, *p*-CA treatment significantly reduced NF-κB expression ($P < 0.05$). *p*-CA significantly reduced the mRNA expression level of the *NF-κB* gene in foam cells compared to ox-LDL and LPS co-treatment ($P < 0.05$) (**Fig. 6B**). Also, Immunofluorescence analysis established the inhibitory effect of *p*-CA acid on ox-LDL and LPS-induced nuclear translocation of p65 (**Fig. 6D**). These results indicate that *p*-CA may serve as potential inflammation inhibitors by reducing inflammation of foam cells in the early stages of atherosclerosis.

DISCUSSION

Cardiovascular diseases are recognized as major causes of death and disability worldwide [47]. In 2019, the World Health Organization reported that these diseases accounted for 32% of the global mortality rate [48]. Meanwhile, according to the 2019 National Health and Nutrition Examination Survey, the prevalence of cardiovascular diseases in South Korea was reported to be 21.8% [49]. The mortality rate from cardiovascular diseases tends to rise sharply with age [50]. Atherosclerosis, a chronic inflammatory vascular disease, has the main characteristic of early lesions in which macrophages ingest ox-LDL to form foam cells [3,51]. Accumulation of lipid-containing foam cells accelerates plaque formation by abnormal cholesterol metabolism and increased inflammation [52]. A previous study reported that LPS treatment in human macrophage-derived foam cells with ox-LDL suppressed cholesterol efflux and increased inflammation [53,54]. Therefore, controlling the balance of cholesterol inflow and outflow to prevent the accumulation of lipids inside macrophages and to inhibit conversion into foam cells is an essential factor in preventing and treating atherogenesis [55]. Research on natural dietary agents such as ferulic acid, resveratrol, and curcumin focuses on foam cell formation inhibition and cholesterol efflux [56-58]. For this reason, it is necessary to improve the knowledge of biological mechanisms to elucidate the health effects of bio active molecules in natural products. *p*-CA is a phenolic compound and mainly has antioxidant and anti-diabetic effects [41,59,60].

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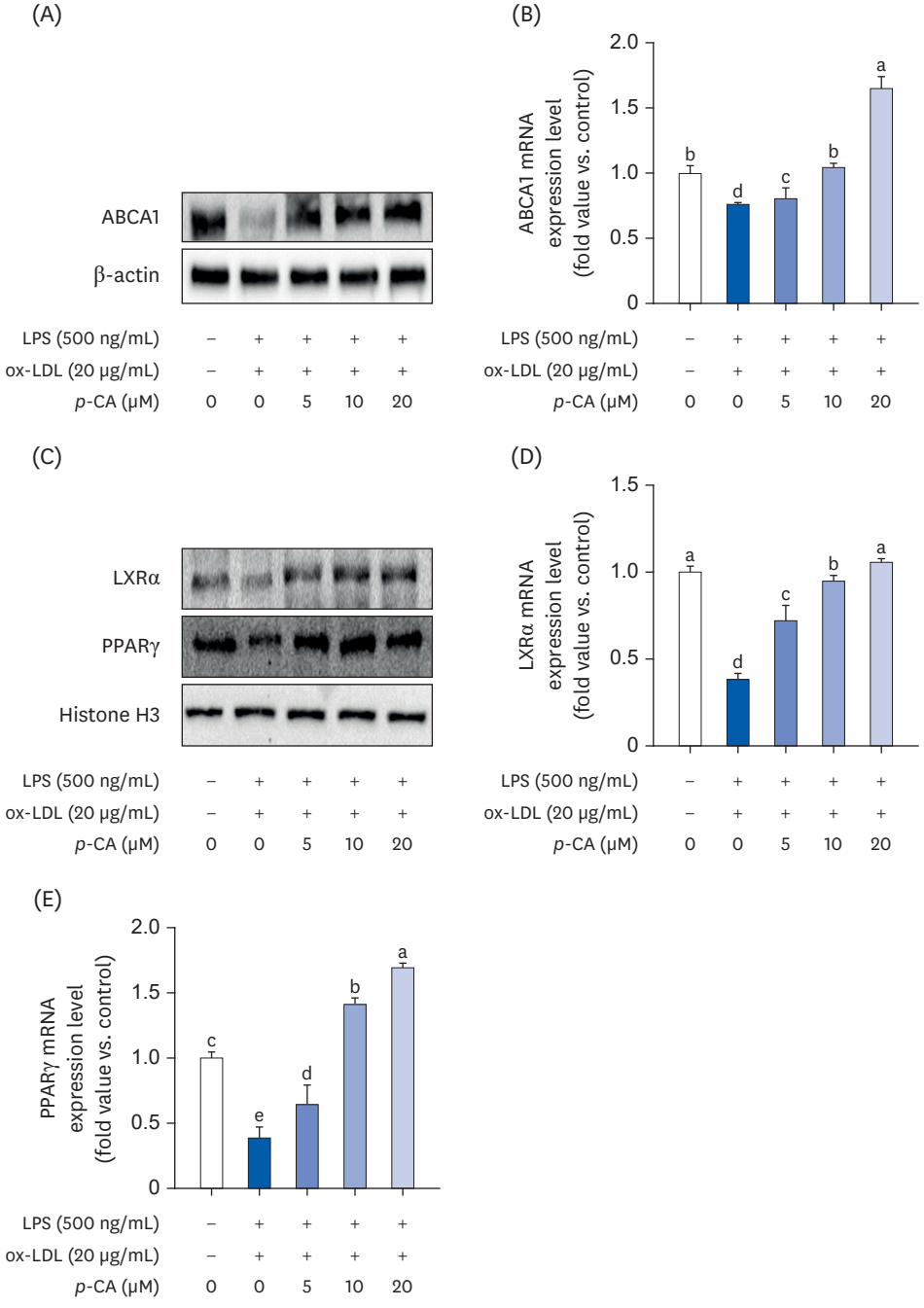

Fig. 4. Upregulation of ABCA1, LXRα, and PPARγ expression by *p*-CA treatment in THP-1 foam cells. The protein expression levels of ABCA1 were determined using (A) immunoblotting. Cells were harvested, and the expression of the (B) ABCA1 mRNA in ox-LDL and LPS-induced foam cells was evaluated. The protein expression levels of LXRα and PPARγ were determined using (C) immunoblotting. Cells were harvested, and the expression of the (D, E) LXRα and PPARγ mRNA in ox-LDL and LPS-induced foam cells was evaluated. (D) LXRα and (E) PPARγ levels. Data are presented as the means ± SD. Data were analyzed by applying the $2^{-\Delta\Delta CT}$ method.
LPS, lipopolysaccharides; ox-LDL, oxidized low-density lipoprotein; *p*-CA, *p*-coumaric acid; ABCA1, ATP binding cassette transporter A1; LXRα, liver X receptor α; PPARγ, Peroxisome proliferator-activated receptor γ.
Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.

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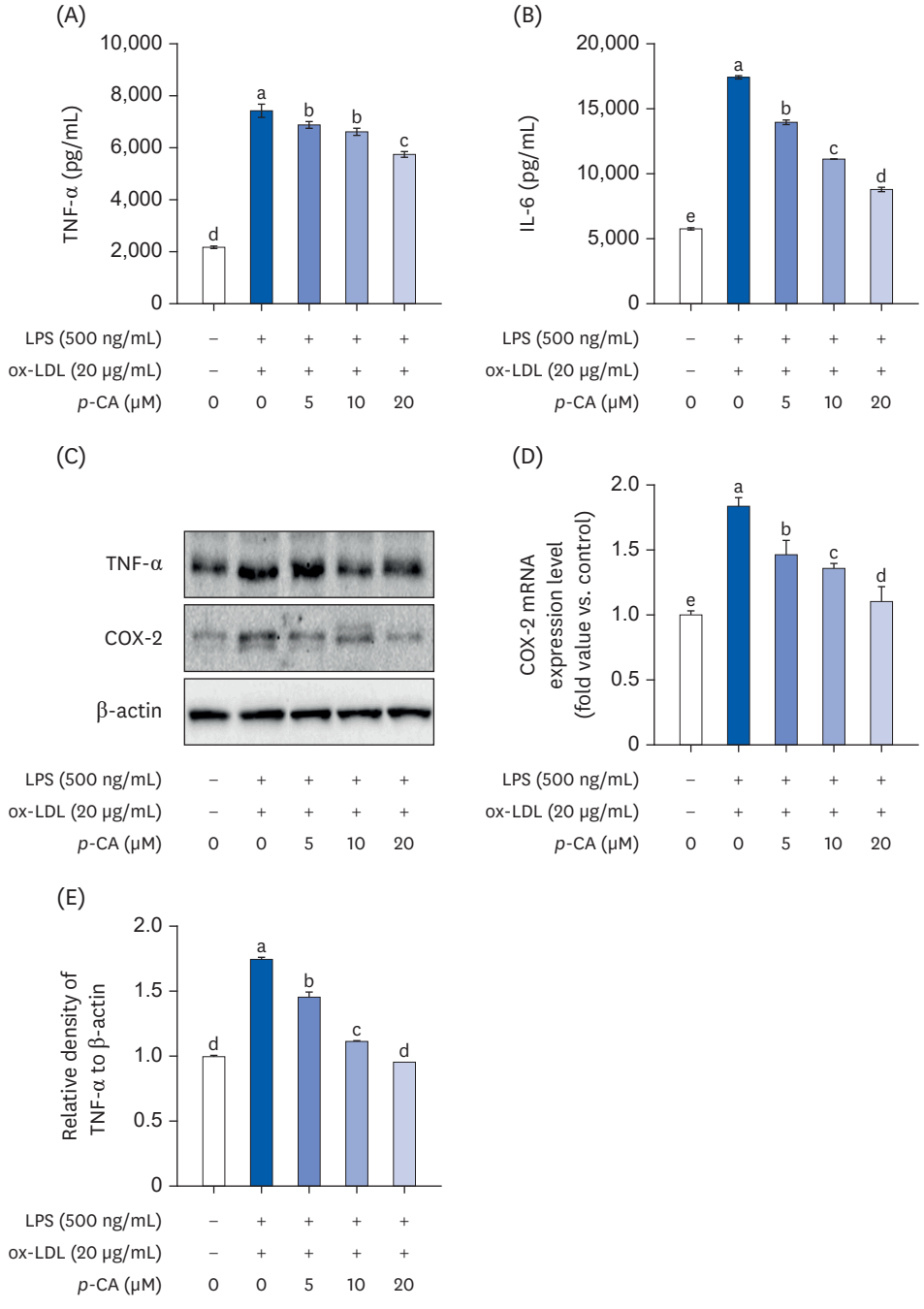

Fig. 5. Inhibition of inflammatory cytokine secretion by *p*-CA treatment in co-treated with ox-LDL and LPS-induced foam cells. THP-1 foam cells were pretreated with different concentrations of *p*-CA (0–20 μM) for 48 h. (A, B) The secretion of IL-6 and TNF-α was measured using an enzyme-linked immunosorbent assay kit. The expression levels of TNF-α and COX-2 were measured using (C) immunoblotting. Cells were harvested, and the expression of the (D) COX-2 mRNA in ox-LDL and LPS-induced foam cells was evaluated. (E) TNF-α levels densities were normalized to β-actin using ImageJ software. Data are presented as the means ± SD.
LPS, lipopolysaccharides; ox-LDL, oxidized low-density lipoprotein; *p*-CA, *p*-coumaric acid; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; COX-2, cyclooxygenase-2.
Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.

The development of atherosclerotic plaques begins with endothelial cell damage, allowing more LDL particles to pass through the vessel wall. These lipoproteins, particularly LDL, become trapped in the intima by the extracellular matrix. The LDL then undergoes

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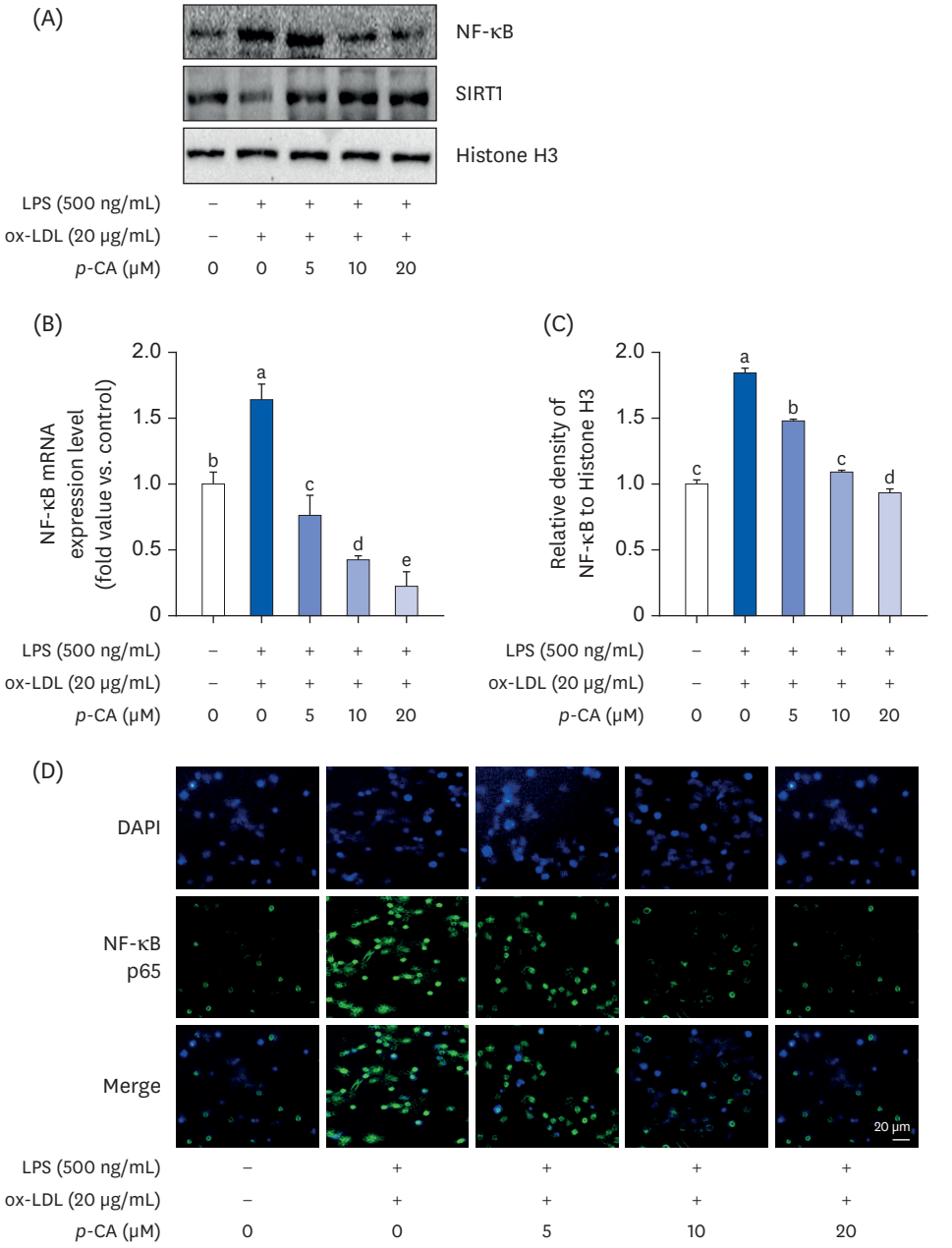

Fig. 6. Inhibition of NF-κB p65 activation by *p*-CA treatment in co-treated with ox-LDL and LPS-induced foam cells. The levels of the NF-κB and SIRT1 proteins were measured using (A) immunoblotting. Cells were harvested, and the expression of the (B) NF-κB mRNA in ox-LDL and LPS-induced foam cells was evaluated. (C) NF-κB levels densities were normalized to β-actin using ImageJ software. Data are presented as the means ± SD. (D) THP-1 foam cells were treated with *p*-CA (0–20 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.
LPS, lipopolysaccharides; ox-LDL, oxidized low-density lipoprotein; *p*-CA, *p*-coumaric acid; NF-κB, nuclear factor-κB; SIRT1, sirtuin 1; DAPI, 4′,6-diamidino-2-phenylindole.
Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.

modification and is taken up by macrophages using specialized cell surface SRs, forming foam cells. As more lipids accumulate, smooth muscle cells migrate to the lesion and encapsulate the plaque, forming a fibrous cap that protects the lipid core from the vessel lumen. These plaques can decrease blood flow or rupture, causing thrombosis and blocking blood flow [61].

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In the early stages of atherosclerosis, LDL has been known as an indicator of the onset of atherosclerosis, and both LDL and total cholesterol are considered major risk factors due to their high correlation with the development of atherosclerosis [62,63]. Khatana *et al.* [64] discovered that the primary mechanism of macrophage formation stems from excessive ox-LDL intake and disruptions in lipid efflux. Macrophages play an essential role in atherosclerosis development, as activated in the subendothelium in atherosclerotic lesions after engulfing LDL [65,66]. Circulating monocytes, recruited to the vascular lamina after LDL uptake, would differentiate into macrophages upon infiltration, and with over-loaded ox-LDL, these macrophages would transform into foam cells, which is a hallmark of atherosclerosis [66]. In other words, the formation of lipid-rich foam cells has a significant impact on the development of atherosclerosis [67]. Lipid-laden foam cells form fibrous atheroma and cause excessive inflammatory responses in endothelial cells. Additionally, ox-LDL is toxic to cells and induces the expression of inflammatory genes, thereby promoting the formation of foam cells. Ox-LDL induced macrophage migration *in vitro* by increasing NF-κB translocation [68]. Additionally, proinflammatory cytokines, including IL-6, COX-2, and TNF-α recruit monocytes to the vascular wall, increase ox-LDL uptake, and increase SR expression, thereby accelerating foam cell formation and atherosclerosis [69-71]. Many recent studies have demonstrated that phytochemicals inhibit ox-LDL uptake by reducing inflammation and the expression of SR-A1, CD36, and LOX-1, thereby attenuating foam cell formation in macrophages [72]. For instance, phenethyl isothiocyanate, sweroside, and eugenol have been shown to reduce macrophage foam cell formation and inflammation by downregulating CD36 expression and upregulating ABCA1 expression [73-75]. Therefore, the mechanisms that inhibit foam cell formation in the early stages of atherosclerosis could ultimately play a crucial role in preventing atherosclerosis and cardiovascular diseases. Our results indicate that the combined treatment of ox-LDL and LPS promotes the accumulation of lipids within cells and increases the amount of Oil red O-stained lipid particles deposited intracellularly. Our data show that a concentration of 20 μM *p*-CA significantly decreased the formation of lipid droplets compared to untreated. We found that Oil red O staining exposed that *p*-CA lowered foam cell formation and lipid uptake, compared with ox-LDL and LPS-treated THP-1 cells. Like this study, Xue *et al.* [76] reported that quercetin reduced lipid accumulation in LPS-induced murine macrophages and inhibited foam cell formation. Liu *et al.* [77] reported that mulberry extract reduced lipid accumulation in ox-LDL-induced foam cells.

Atherosclerosis is a chronic inflammatory condition, and many studies have shown that various cytokines are essential in the advancement of atherosclerosis and the instability of plaques [78-80]. IL-6 is a cytokine that regulates the inflammatory response of leukocytes and other cells and is also considered to be a biomarker of inflammation [79]. TNF-α is deemed an effective pro-inflammatory mediator, which promotes the expression of different inflammatory cytokines and adhesion molecules and increases the apoptosis of VSMCs, thus promoting atherosclerosis and plaque instability [80]. NF-κB, a critical signaling pathway that affects the entire process of atherosclerosis from inflammatory response and plaque formation to rupture, increases cytokines expression and chemokines accelerates foam cell creation [81]. Recent cells and animal studies have exposed that inhibiting NF-κB, a regulator of macrophage inflammation, reduces foam cell formation [70,82,83]. Here, we confirmed that co-treatment on LPS and ox-LDL upregulated the protein expression of NF-κB, TNF-α, and COX-2 and reduced SIRT1 compared to the untreated cells. By contrast, *p*-CA treatment declined the expression of NF-κB, TNF-α, and COX-2 and upregulated SIRT1. Lin *et al.* [84] reported that baicalein suppressed inflammation through NF-κB signaling in ox-LDL-induced endothelial umbilical vein cells. Kuo *et al.* [85] reported that ellagic acid inhibited

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inducible nitric oxide synthase, IL-8 and IL-6 production through NF-κB and MAPK pathway in ox-LDL stimulated human umbilical vein endothelial cells. These findings suggest that *p*-CA suppresses ox-LDL and LPS-induced inflammation by regulating NF-κB expression. Foam cells are formed by lipid uptake and the loss of cholesterol homeostasis, where the absorption of ox-LDL by monocyte-derived macrophages is an early event in atherosclerosis [86]. When monocytes ingest lipoprotein particles and transform into macrophages, an imbalance arises between cholesterol uptake and efflux, resulting in the creation of foam cells [87]. Therefore, balancing cholesterol influx and efflux in macrophages is important to prevent lipid overload and atherosclerotic plaque formation. Many studies using natural compounds have recently been conducted to control the cholesterol mechanism.

Promoting cholesterol efflux to prevent excessive lipid accumulation is essential in avoiding macrophage-derived foam cell creation in atherosclerosis [88]. Cholesterol efflux is necessary as it activates extracellular cholesterol receptors like ApoA1 and HDL through membrane transporters such as ABCA1, ABC transporters are crucial in mediating excessive cholesterol removal from cells to maintain cholesterol homeostasis [19,89]. ABCA1, a membrane transporter abundant in macrophages, generates precursors for HDL particles by promoting the transfer of cholesterol and phospholipids from lipid-rich macrophages to lipid-free apoA1 [90]. In animal and human experiments, loss of ABCA1 or induced excessive cholesterol deposition in macrophages, causing an inflammatory response [91,92]. Recent studies have reported that the ABC transporter, a cholesterol efflux pathway, suppresses atherosclerosis by forming HDL [93]. LXRα and LXRβ are essential for protecting against cardiovascular diseases [94]. Members of the nuclear receptor superfamily of DNA-binding transcription factors, cholesterol sensor LXRs, promote reverse cholesterol transport, bile acid synthesis, and intestinal cholesterol efflux [95]. LXR ligand activation strongly upregulates gene expression of ABCG1 and ABCA1, triggering the cholesterol efflux pathway [96]. LXR-deficient mice accelerated atherosclerosis by accumulating cholesterol, whereas curcumin-fed mice inhibited the development of atherosclerosis by activating LXRα [97]. Studies in macrophages have shown that PPARγ regulates cholesterol influx, efflux, and metabolism. Activated PPARγ promotes ApoA1-mediated cholesterol efflux, inhibits the formation of macrophage-derived foam cells and reduces the accumulation of triglycerides in treated macrophages. PPARγ, which mediates adipogenesis, is primarily expressed in the heart, macrophages, and adipocytes and is associated with lipid accumulation in the liver, heart, and blood vessels [98,99]. In macrophages, PPARγ has been shown to increase ABCA1 levels and upregulate LXR expression [22,100]. It has been demonstrated that PPARγ activation can induce cholesterol clearance and ABCA1/G1 in macrophages via LXR-mediated transcriptional regulation [100]. Therefore, the interaction between ABCA1/LXR/PPAR is essential in maintaining cholesterol homeostasis and HDL metabolism. Franceschelli *et al.* [101] reported that hydroxytyrosol reduced foam cell formation and inflammation by regulating the PPAR/LXRα/ABCA1 pathway. Zhao *et al.* [102] reported that betulinic acid promoted ABCA1 and suppressed NF-κB in LPS-treated THP-1 cells. Yan *et al.* [103] reported that curcumin increased cholesterol efflux via ABCA1/PPARγ in LPS and IFNγ induced murine macrophage. Therefore, we discuss the ABCA1/ LXRα/ PPARγ agonists involved in regulating cholesterol efflux.

The SRs composed of CD36, SR-A, and LOX-1, identified for their role in mediating the uptake and internalization of modified lipoproteins, such as oxidized LDL, have generally been considered essential for foam cell formation [104]. LOX-1, expressed in macrophages and smooth muscle cells, is a transmembrane glycoprotein in endothelial cells that binds

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to and internalizes ox-LDL [105]. LOX-1, the major ox-LDL receptor on macrophages, is induced by proinflammatory cytokine stimulation [106]. Recent animal and cell studies have reported that downregulation of LOX-1 expression inhibited atherosclerotic lesion formation and reduced inflammation [107,108]. Among SRs, SR-A and CD36 regulate inflammatory signaling pathways, including those that lead to macrophage foam cell formation, lesion macrophage apoptosis, and plaque necrosis during atherosclerosis [109]. CD36 and SR-A mediate the transfection of ox-LDL, accumulating lipids and promoting the production of NF-κB and inflammatory cytokines [110]. In a recent study, knockout mice of CD36 declined ox-LDL uptake, foam cell, and plaque formation in mice [111]. Li *et al.* [112] reported that paeonol inhibits lipid accumulation in macrophages by downregulating CD36. Wang *et al.* [113] reported that ganoderic acid A attenuated ox-LDL-mediated foam cell creation by reducing LOX-1 and CD36 in THP-1 cells. Shen *et al.* [114] conveyed that bergaptol inhibited foam cell creation by decreasing CD36 and SR-A1 in LPS and ox-LDL-mediated murine macrophages. Therefore, we focused on the mechanisms of foam cell formation inhibition by concentrating on the ABCA1/LXR/PPARγ signaling pathway related to cholesterol efflux and the CD36/LOX-1/SR-A1 signaling pathway related to lipid accumulation. Similar to previous research, our study found that *p*-CA increases cholesterol efflux by inducing ABCA1/LXRα/PPARγ in THP-1 macrophages co-treated with ox-LDL and LPS while inhibiting lipid uptake by increasing SR-A1, CD36, and LOX-1.

Altogether, *p*-CA inhibits the formation of foam cells induced by co-treatment of ox-LDL and LPS in THP-1-derived macrophages. Foam cell formation, along with the increase in inflammatory cytokine production, NF-κB and target gene expression, is inhibited by *p*-CA in foam cells. These results demonstrate that *p*-CA is effective natural substances for preventing and treating atherosclerosis.

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