

PCSK9 inhibitor Evolocumab alleviates atherosclerosis progression by suppressing endothelial cell injury *via* the ROS/MAPK pathway

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Abstract. Atherosclerosis (AS) progression is closely linked to oxidized low-density lipoprotein (Ox-LDL)-induced endothelial cell injury. This study investigates the molecular mechanism by which the PCSK9 inhibitor Evolocumab (EVC) alleviates AS. We used Ox-LDL-induced HUVECs and high-fat diet (HFD)-fed ApoE^{−/−} mice as *in vitro* and *in vivo* AS models. AS plaque formation was detected by HE and Oil Red O staining. The levels of lipid indicators and oxidative stress-related factors were measured *via* ELISA. The expression levels of PCSK9, apoptosis-related proteins, and signaling pathway-related proteins were determined *via* Western blotting. The result shows that EVC improved blood lipids and alleviated aortic injury in atherosclerotic mice, and concomitantly suppressed Ox-LDL-induced apoptosis and oxidative stress in HUVECs. In terms of the molecular mechanism, EVC attenuated the Ox-LDL-induced phosphorylation of ERK1/2, JNK and p38. Both the ROS inhibitor N-acetylcysteine and the p38 MAPK signaling pathway inhibitor SB203580 can enhance the suppressive effect of the PCSK9 inhibitor EVC on Ox-LDL induced apoptosis and oxidative stress in HUVECs, while the p38 MAPK signaling pathway activator C16-PAF shows the opposite results. In conclusion, the PCSK9 inhibitor EVC inhibits Ox-LDL-induced HUVECs apoptosis and oxidative stress by inhibiting the ROS/MAPK signaling pathway, thereby alleviating the progression of AS.

Key words: Atherosclerosis — PCSK9 inhibitor — Evolocumab — MAPK — Endothelial cells

Introduction

Atherosclerosis (AS) is a chronic inflammatory vascular disease (Fan and Watanabe 2022). Globally, cardiovascular diseases (CVD) are the leading cause of human mortality, with AS being a major driver of its onset and progression (Chong et al. 2025). The study highlights that the broader societal and economic impacts of AS include continuously rising medical expenditures, challenges in healthcare accessibility, and a significant decline in patients' quality of life. Furthermore, projections indicate that by 2035, the economic burden attributable to AS-induced CVD in the United States

alone is expected to reach approximately \$509 billion (Khera et al. 2020). The development of AS involves complex processes such as endothelial cell dysfunction, inflammation, smooth muscle cell proliferation, and macrophage activation (Ling et al. 2021). Oxidized low-density lipoprotein (Ox-LDL)-induced endothelial dysfunction is one of the leading causes of the pathogenesis of AS (Huo et al. 2022). Ox-LDL promotes the formation and development of atherosclerotic plaques by inducing vascular endothelial inflammation, apoptosis, and destruction of antioxidant ability (Zhao et al. 2020). Therefore, protecting endothelial cell function may be a promising method to treat AS.

Endothelial cells (ECs) are monolayers of cells on the surface of blood vessels that play critical roles in regulating vascular permeability and maintaining vascular homeostasis (Claesson-Welsh et al. 2021). Previous studies have shown that reactive oxygen species (ROS) accumulation can induce AS-related endothelial dysfunction (Jiang et al. 2022). Dur-

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ing AS, the production of ROS is significantly increased, and the antioxidant system is downregulated, eventually leading to oxidative stress (Batty et al. 2022). In addition, ROS overproduction can lead to the sequential phosphorylation of a series of protein kinases, tyrosine kinases and mitogen-activated protein kinases (MAPK), including the phosphorylation and activation of ERK1/2, JNK and p38 MAPK (Zhang et al. 2016), and the inhibition of P38 MAPK phosphorylation can reduce Ox-LDL-induced inflammatory injury to vascular endothelial cells (Kuang et al. 2021), revealing that the MAPK signaling pathway plays an important role in the regulation of endothelial cell function. However, the specific mechanisms by which ROS-mediated MAPK activation contributes to vascular endothelial injury-particularly under Ox-LDL stimulation-remain incompletely elucidated, and the cross-talk between different MAPK subtypes and their downstream targets in this context has not been fully defined. Therefore, this study aims to clarify the precise role and molecular details of the ROS/MAPK signaling pathway in Ox-LDL-induced injury in HUVECs, thereby addressing this identified knowledge gap.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a soluble protease that plays an important role in the regulation of cholesterol homeostasis and cardiovascular diseases such as AS (Sundararaman et al. 2021). Previous studies have shown that PCSK9 increases the level of low-density lipoprotein cholesterol (LDL-C) and regulates blood lipid levels by promoting the degradation of low-density lipoprotein receptor (LDLR) (Shin et al. 2024). In addition, PCSK9 is involved in the production of inflammatory cytokines, endothelial dysfunction, and the formation of atherosclerotic plaques (Wu et al. 2022). An increased PCSK9 concentration can accelerate endothelial apoptosis and reduce endothelial function (Sotler and Šebešćin 2023). Clinical studies have shown that the inhibition of PCSK9 can attenuate AS generation and plaque inflammation (Karagiannis et al. 2018). PCSK9 inhibitors are a class of lipid-lowering drugs that not only reduce plasma LDL-C levels but also reduce the risk of cardiovascular events (Yang et al. 2023). Currently clinically applied PCSK9 inhibitors include Alirocumab and Evolocumab (EVC), both of which are fully human monoclonal antibodies (mAbs) that specifically bind to PCSK9, blocking its interaction with LDL receptors (Roth and Davidson 2018). According to reports, Alirocumab and EVC can reduce LDL-C levels by up to 60% and have been proven to be relatively safe (Dixon et al. 2019). Furthermore, studies have shown that PCSK9 inhibitors significantly reduce all-cause mortality and healthcare utilization in patients with hyperlipidemia compared to statins (Huang et al. 2024). However, although the lipid-lowering efficacy of PCSK9 inhibitors is well established, the molecular mechanisms underlying their potential protective effects against AS beyond LDL reduction-particularly the direct

effects of EVC on vascular endothelial cell function, plaque stability, and the inflammatory microenvironment-remain poorly understood and require further investigation. This study aims to explore whether EVC alleviates the progression of AS by modulating ROS/MAPK signaling to reduce endothelial cell injury.

Therefore, in this study, we will perform experiments at the cell and animal levels to explore in depth the mechanism of action of the PCSK9 inhibitor EVC in AS and provide new insights for the treatment of AS.

Materials and Methods

Cell culture and treatment

Human umbilical vein endothelial cell line (HUVECs) serve as a classic endothelial cell model in cardiovascular research due to their highly conserved biological characteristics across various vascular beds. Therefore, we selected HUVECs to simulate Ox-LDL-induced endothelial injury – a key pathological process in the early stages of atherosclerosis. HUVECs was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, USA) and 100 U/ml penicillin/streptomycin (Sigma, USA) in a humidified incubator at 37°C with 5% CO₂. As mentioned previously (Xia et al. 2019), HUVECs were treated with Ox-LDL (100 µg/ml; MP6009, MKBio, China) for 24 h to induce the AS cell model. HUVECs were treated with different concentrations (0, 0.1, 1, 10, or 100 µg/ml) of EVC (Safaeian et al. 2019) (HY-P9930; MedChem Express, USA) for 24 h to explore the toxic effects of EVC on HUVECs. To study the protective effect of EVC on Ox-LDL-induced HUVECs damage, the cells were cotreated with 100 µg/ml Ox-LDL and 10 µg/ml EVC for 24 h.

To explore the effects of the ROS/MAPK signaling pathway on Ox-LDL-induced HUVECs apoptosis and oxidative stress, HUVECs were treated with 1 mM of the ROS inhibitor N-acetylcysteine (NAC, A0150000, Sigma-Aldrich, USA), 10 µM of the p38 MAPK signaling pathway inhibitor SB203580 (HY-10256, Med Chem Express, USA) or 5 µM of the p38 MAPK signaling pathway activator C16-PAF (HY-108635, MedChemExpress, USA) for 1 h and then treated with Ox-LDL (100 µg/ml) and EVC (10 µg/ml) for 24 h.

Construction of the AS animal model

ApoE^{-/-} mice lack apolipoprotein E, resulting in disordered lipid metabolism. When fed a high-fat diet (HFD), these mice spontaneously develop the full spectrum of AS lesions, from fatty streaks to advanced plaques, with pathological features closely resembling human AS. Therefore, they

were selected as the animal model for this study. Fifteen 6-week-old male homozygous ApoE^{-/-} mice (weighing approximately 18–20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd and the mice were housed at a temperature of 22 ± 2°C, a relative humidity of 55 ± 15%, noise <60 dB, and a light/dark cycle of 12–12 h in pathogen-free facilities for one week. The mice were then randomly divided into 3 groups ($n = 5/\text{group}$): the control group, the AS group, and the AS+EVC group. ApoE^{-/-} mice were fed a HFD containing 0.25% cholesterol and 15% cocoa butter for 12 weeks to establish the AS animal model. The mice in the control group were fed a conventional diet. Mice in the AS+EVC group were fed a HFD for 12 weeks, followed by subcutaneous injections of 30 mg/kg EVC (Yang et al. 2023) administered every two weeks over an 8-week period, for a total of four injections. The mice in the control group and the AS group were injected with the same volume of saline at the corresponding time points. All the mice were subsequently euthanized. After the heart stopped, the aortic root tissue was removed for subsequent experiments.

Detection of blood lipid indicators

Serum was collected after blood samples were centrifuged at 1300×g for 10 min at 4°C. The total cholesterol (T-CHO) assay kit (A111-1-1, Nanjing Jiancheng Bioengineering Institute, China), triglyceride (TG) test kit (A110-1-1, Nanjing Jiancheng Bioengineering Institute, China), low-density lipoprotein cholesterol (LDL-C) test kit (A113-2-1, Nanjing Jiancheng Bioengineering Institute, China), and high-density lipoprotein cholesterol (HDL-C) test kit (A112-2-1, Nanjing Jiancheng Bioengineering Institute, China) were used to determine the contents of serum TC, TG, LDL-C, and HDL-C.

ELISA

Serum was collected as described above. PCSK9 content was determined *via* a Human Proprotein Convertase 9/PCSK9 ELISA kit (EH384RB, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, USA), and the concentration of PCSK9 in the samples was quantified based on a standard curve.

CCK-8

Cell viability was determined *via* a CCK-8 kit (C0037, Beyotime, China). HUVECs were seeded in 96-well plates (4×10^4 cells/well) and treated with EVC (0, 0.1, 1, 10, or 100 µg/ml) or Ox-LDL for 24 h. Then, 10 µl of CCK-8 solution was added to each well and incubated at 37°C for 2 h, and the absorbance of the cells at 450 nm was measured *via* a microplate reader (Bio-Rad, USA).

Apoptosis detection by flow cytometry

The percentage of apoptotic cells was determined *via* flow cytometry *via* an Annexin V-FITC apoptosis detection kit (C1062S, Beyotime, China). Briefly, HUVECs subjected to different treatments were resuspended at a density of 5×10^5 cells/ml. Then, 5 µl of Annexin V-FITC and 10 µl of PI solution were added to the cells, mixed gently, and incubated in the dark for 15 min. The percentages of Annexin V-FITC-positive/PI-negative cells and Annexin V-FITC-positive/PI-positive cells were analyzed *via* flow cytometry (FACSCalibur, USA).

Western blot analysis

Total protein was extracted from aortic tissues or HUVECs using RIPA lysis buffer (P0038, Beyotime, China) containing a protease and phosphatase inhibitor cocktail (P1045, Beyotime, China). Protein concentration was determined with a BCA protein assay kit (P0010, Beyotime, China). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. After membrane was blocked with 5% skim milk for 1 h at room temperature, the membrane was incubated with primary antibody Bcl-2 (1:1000, ab182858, Abcam, UK), Bax (1:2000, 50599-2-Ig, Proteintech, USA), cleaved-caspase3 (1:2000, 82707-13-RR, Proteintech, USA), PCSK9 (1:1000, PA5-87122, Thermo Fisher Scientific, USA), p38 (1:1000, 14064-1-AP, Proteintech, USA), p-p38 (1:2000, 28796-1-AP, Proteintech, USA), ERK1/2 (1:2000, #4696, Cell Signaling Technology, USA), p-ERK1/2 (1:2000, # 4370, Cell Signaling Technology, USA), JNK (1:1000, #67096, Cell Signaling Technology, USA), p-JNK (1:1000, #9251, Cell Signaling Technology, USA), β-actin (1:1000, ab8226, Abcam, UK) at 4°C overnight. Then, the membrane was incubated with the corresponding secondary antibody for 2 h at room temperature. The protein bands were visualized *via* an enhanced chemiluminescence detection kit (P0018, Beyotime, China) and analyzed *via* ImageJ software.

Immunofluorescence

After HUVECs were stimulated with 100 µg/ml Ox-LDL for 24 h, the cells were fixed with 4% formaldehyde for 30 min. Then, the cells were incubated with 0.1% Triton X-100 for 30 min at room temperature, and the cells were blocked with goat serum for 1 h at room temperature. After being washed with PBS, the cells were incubated with a PCSK9 antibody (1:200, PA5-87122, Thermo Fisher Scientific, USA) at 4°C overnight. Then, the samples were incubated with an Alexa Fluor 488-labeled goat anti-rabbit IgG (H+L) secondary antibody (1:1000, ab150077, Abcam, UK) at room temperature in the dark for 2 h. Nuclear staining was performed *via*

DAPI (P0131, Beyotime, China). Finally, the images were observed and imaged under a fluorescence microscope (IX3, Olympus, Japan).

Histological and immunohistochemical examinations

Mouse aorta tissues were collected, fixed in 4% paraformaldehyde for 24 h, fractionally dehydrated *via* a series of alcohols (70, 80, 90, 95 and 100%), embedded in paraffin and cut into 5 μ m thick sections. AS lesions were detected *via* a HE staining kit (G1120; Solarbio, China), and lipid deposition was evaluated *via* an Oil Red O staining kit (D027-1-3; Nanjing Jiancheng Bioengineering Institute, China).

For immunohistochemistry, the sections were routinely deparaffinized, rehydrated and heated in 0.01 M sodium citrate buffer. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide and 10% goat serum for 1 h. The sections were then incubated with an anti-PCSK9 antibody (1:100, PA5-87122, Thermo Fisher Scientific, USA) at 4°C overnight and then with an HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:4000, ab205718, Abcam, UK) for 1 h, and the sections were stained with a DAB chromogenic kit (DA1010, Solarbio, China) and a light microscope (X81, Olympus, Japan) was used for visualization and imaging.

ROS detection

The production of ROS was assessed by measuring the fluorescence intensity of dihydroethidium (DHE, D7008, Sigma-Aldrich, USA) and 2',7'-dichlorofluorescein diacetate (DCFDA, 287810, Sigma-Aldrich, USA). Briefly, 5 μ m thick sections of the aortic root were stained with DHE solution and DAPI (nuclear) according to standard laboratory procedures. In addition, HUVECs were seeded in six-well plates. After drug treatment, the cells were washed with PBS, and then DCFDA at a concentration of 20 μ M was added to the DMEM for incubation for 20 min. Finally, images were acquired under a microscope.

Detection of SOD, MDA, and GSH

The levels of SOD, MDA, and GSH in mouse aortic tissues or HUVECs were measured according to the instructions of the respective assay kits: SOD assay kit (A001-3-1, Nanjing Jiancheng Bioengineering Institute, China), MDA assay kit (A003-1-1, Nanjing Jiancheng Bioengineering Institute, China), and GSH assay kit (A006-2-1, Nanjing Jiancheng Bioengineering Institute, China). Briefly, the tissues or cells were homogenized or lysed, followed by centrifugation to collect the supernatant. The working solutions were then added to the samples to determine the content of each parameter.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0, with all measurement data expressed as mean $\pm$ standard deviation (mean $\pm$ SD). Prior to analysis, the normality of data distribution was verified using the Shapiro-Wilk test. For normally distributed data, differences between two groups were analyzed using Student's *t*-test, while comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. In cases where data failed the normality test, non-parametric tests were employed: the Mann-Whitney U test for two-group comparisons and Kruskal-Wallis test with Dunn's *post hoc* test for multiple group comparisons $p < 0.05$ was considered statistically significant.

Results

PCSK9 inhibitor EVC relieve the progression of AS

We first detected the effect of the PCSK9 inhibitor EVC on the progression of AS. Compared with those in the control group, the serum TC, TG, and LDL-C levels of the mice in the AS group were significantly greater, and the HDL-C level was significantly lower. EVC injection reversed the effect of AS (Fig. 1A–D). The PCSK9 levels in the serum were measured *via* ELISA. Compared with those in the control group, the serum PCSK9 level was significantly increased in the AS group, and the PCSK9 level was significantly reduced after EVC injection (Fig. 1E). These results indicate that the PCSK9 inhibitor EVC can reduce the lipid levels of AS mice and attenuate the progression of AS.

Ox-LDL induces apoptosis and oxidative stress in HUVECs and upregulates the expression of the PCSK9 protein

Ox-LDL-induced endothelial cell injury is closely related to AS, and endothelial cell injury is usually characterized by apoptosis and increased ROS production (Gong et al. 2019). Therefore, we analyzed Ox-LDL-induced endothelial cell damage and PCSK9 protein expression. First, the results of the CCK-8 and flow cytometry analyses revealed that, compared with the control group, the Ox-LDL treatment group had significantly reduced cell viability and an increased apoptosis rate (Fig. 2A,B). The results of Western blot analysis revealed that, compared with those in the control group, the protein expression of Bax and cleaved-caspase-3 was significantly upregulated, whereas the protein expression of Bcl-2 was significantly downregulated in the Ox-LDL treatment group (Fig. 2C). The results of the analysis of oxidative stress-related indicators revealed that in the Ox-LDL treatment group, the contents of ROS and MDA were significantly increased,

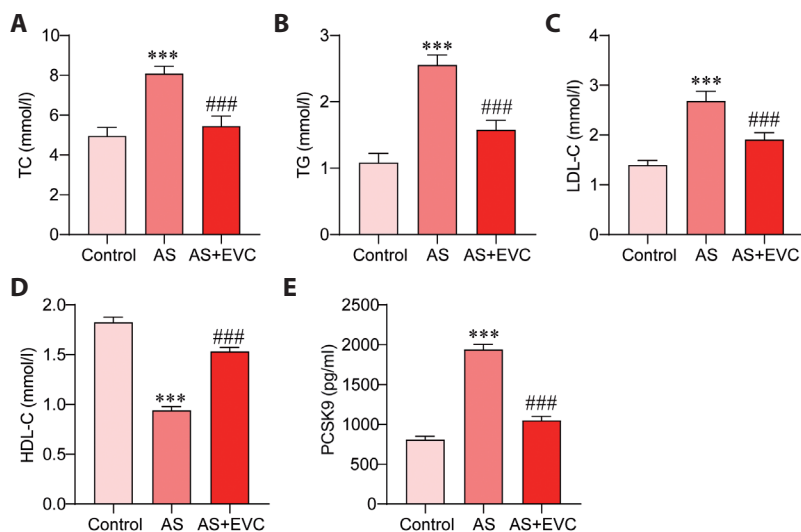


Figure 1. PCSK9 inhibitor Evolocumab attenuated the progression of AS. **A.** TC levels were measured using a kit. **B.** TG levels were measured using a kit. **C.** LDL-C levels were measured using a kit. **D.** HDL-C levels were measured using a kit. **E.** PCSK9 level in mouse serum detected by ELISA. *** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. AS, atherosclerosis group; AS+EVC, atherosclerosis + Evolocumab group.

whereas the contents of SOD and GSH were significantly reduced (Fig. 2D,E). In addition, the results of Western blot and immunofluorescence detection of PCSK9 protein expression revealed that PCSK9 protein expression in the Ox-LDL-treated group was significantly greater than that in the control group (Fig. 2F,G). These results indicate that Ox-LDL induces apoptosis and oxidative stress in HUVECs and upregulates the expression of the PCSK9 protein.

PCSK9 inhibitor EVC inhibited Ox-LDL-induced HUVECs apoptosis and oxidative stress

The above experiments confirmed that Ox-LDL-induced HUVECs damage was correlated with the abnormal expression of PCSK9. We further detected the effects of PCSK9 inhibitors EVC on Ox-LDL-induced HUVECs apoptosis and oxidative stress. First, we detected the effects of EVC at different concentrations (0, 0.1, 1, 10, and 100 $\mu\text{g/ml}$) on HUVECs cytotoxicity *via* a CCK-8 assay. The results showed that 0–100 $\mu\text{g/ml}$ of EVC did not exert any toxic effects on HUVECs, and 100 $\mu\text{g/ml}$ of EVC significantly enhanced cell viability (Fig. 3A). Furthermore, we also found that EVC at 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ could significantly improve the viability of HUVECs after Ox-LDL treatment (Fig. 3B). Therefore, EVC at a low effective concentration of 10 $\mu\text{g/ml}$ was chosen for subsequent experiments. The results of Western blot analysis revealed that, compared with the Ox-LDL group, the EVC treatment group presented significantly lower expression levels of the PCSK9 protein (Fig. 3C). The flow cytometry results revealed that the percentage of apoptotic cells in the EVC treatment group was significantly lower than that in the Ox-LDL group (Fig. 3D). The results of Western blot detection of the expression of the apoptosis-related proteins Bcl-2, Bax and cleaved-caspase-3 revealed that EVC

treatment reversed the regulatory effect of Ox-LDL on these proteins, resulting in the upregulation of Bcl-2 protein expression and the downregulation of Bax and cleaved-caspase-3 protein expression (Fig. 3E). In addition, the results of oxidative stress-related indicator tests showed that EVC treatment weakened the effects of Ox-LDL, leading to a significant reduction in ROS and MDA levels, as well as a significant increase in SOD activity and GSH content (Fig. 3F,G). In summary, the PCSK9 inhibitor EVC significantly inhibited Ox-LDL-induced HUVECs apoptosis and oxidative stress.

PCSK9 inhibitor EVC exerts its effects by inhibiting the ROS/MAPK signaling pathway

Previous studies have indicated that the activation of the p38 MAPK signaling pathway is involved in Ox-LDL-induced HUVECs apoptosis and ROS generation (Zhang et al. 2016). We further detected proteins related to the p38 MAPK signaling pathway under PCSK9 inhibitor treatment. Western blot analysis revealed that compared to the control group, the levels of p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK were significantly increased in the Ox-LDL-treated group, while the addition of EVC partially attenuated the effects induced by Ox-LDL (Fig. 4A). These results indicated that PCSK9 inhibitor EVC effectively inhibited the abnormal activation of the ROS/MAPK pathway under Ox-LDL treatment.

PCSK9 inhibitor EVC suppresses Ox-LDL-induced apoptosis and oxidative stress in HUVECs by inhibiting the ROS/MAPK signaling pathway

To further verify whether the PCSK9 inhibitor EVC inhibits Ox-LDL-induced HUVECs apoptosis and oxidative stress

through the inhibition of the ROS/MAPK pathway *in vitro*, we added NAC, SB203580 and C16-PAF to perform experiments. Western blot analysis indicated that, compared to EVC treatment alone, the addition of NAC or SB203580 significantly suppressed the levels of p-p38/p38, p-ERK/ERK, and p-JNK/JNK, whereas supplementation with C16-PAF markedly increased their expression (Fig. 5A). The results of the CCK-8 and flow cytometry analyses revealed that, compared with that after EVC treatment, the cell viability was further increased and the apoptosis rate was further reduced after the addition of NAC or SB203580. The addition of C16-PAF had the op-

posite effect (Fig. 5B,C). The results of Western blot analysis revealed that the protein expression of Bcl-2 was upregulated and that the protein expression of Bax and cleaved-caspase-3 was downregulated in the EVC treatment group. This effect became more significant after the addition of NAC or SB203580; C16-PAF treatment reversed the effect of EVC (Fig. 5D). Compared with those in the Ox-LDL group, the ROS and MDA levels were significantly lower, and the SOD and GSH contents were significantly greater in the EVC-treated group. This effect was more significant after the addition of NAC or SB203580, whereas in the C16-PAF treatment group,

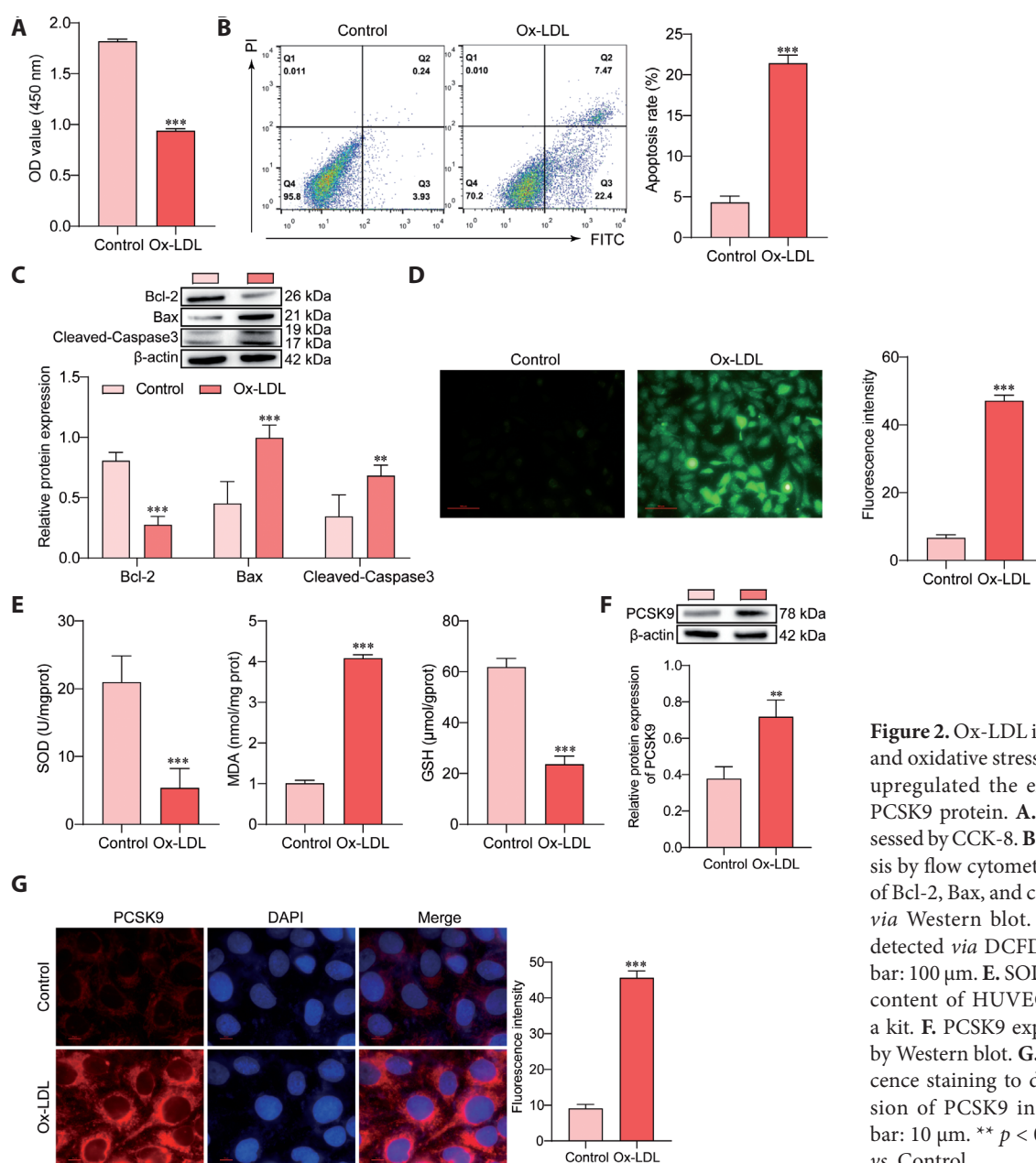


Figure 2. Ox-LDL induced apoptosis and oxidative stress in HUVECs and upregulated the expression of the PCSK9 protein. **A.** Cell viability assessed by CCK-8. **B.** Apoptosis analysis by flow cytometry. **C.** Expression of Bcl-2, Bax, and caspase-3 detected *via* Western blot. **D.** ROS content detected *via* DCFDA staining, scale bar: 100 μ m. **E.** SOD, MDA and GSH content of HUVECs measured *via* a kit. **F.** PCSK9 expression analyzed by Western blot. **G.** Immunofluorescence staining to detect the expression of PCSK9 in HUVECs, scale bar: 10 μ m. ** $p < 0.01$, *** $p < 0.001$ vs. Control.

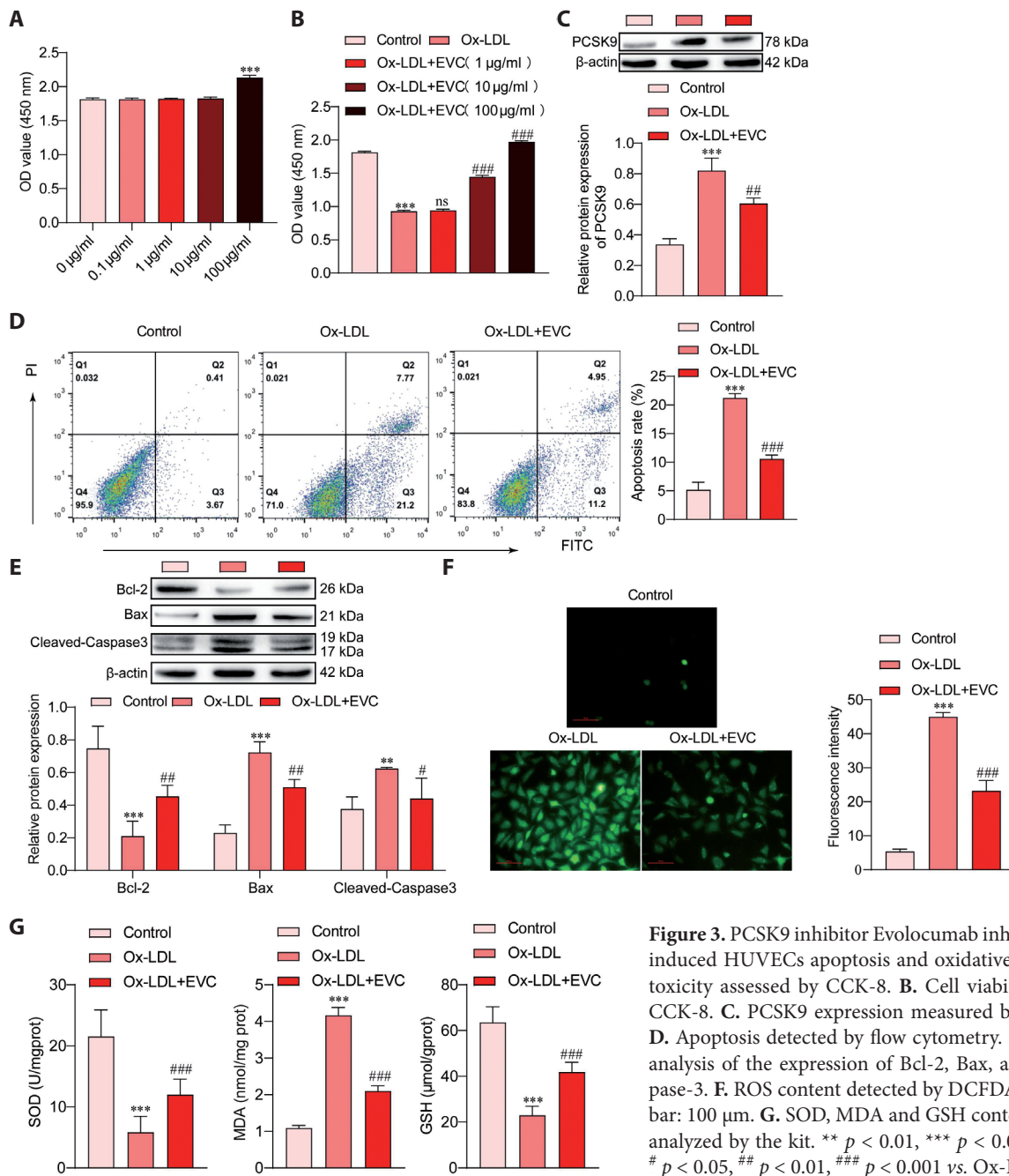


Figure 3. PCSK9 inhibitor Evolocumab inhibited Ox-LDL-induced HUVECs apoptosis and oxidative stress **A.** Drug toxicity assessed by CCK-8. **B.** Cell viability assessed by CCK-8. **C.** PCSK9 expression measured by Western blot. **D.** Apoptosis detected by flow cytometry. **E.** Western blot analysis of the expression of Bcl-2, Bax, and cleaved-caspase-3. **F.** ROS content detected by DCFDA staining, scale bar: 100 µm. **G.** SOD, MDA and GSH content of HUVECs analyzed by the kit. ** $p < 0.01$, *** $p < 0.001$ vs. Control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Ox-LDL.

the effect of EVC was partially reversed (5E,F). These results showed that the PCSK9 inhibitor EVC suppressed Ox-LDL-induced HUVECs apoptosis and oxidative stress through the inhibition of the ROS/MAPK pathway.

PCSK9 inhibitor EVC alleviates the progression of AS in mice

Finally, we validated the alleviating effect of the PCSK9 inhibitor EVC on AS mice *in vivo*. Oil Red O staining re-

sults showed that no intimal thickening or bright red AS areas were observed in the aortas of control group mice. In contrast, the AS group exhibited significant aortic intimal thickening with apparent bright red AS lipid deposits. Compared to the AS group, EVC treatment markedly reduced aortic intimal thickening and decreased the area of AS lipid regions (Fig. 6A). The results of HE staining revealed that the structure of the aorta was normal, the intimal thickness of the vessels was not thickened, and the formation

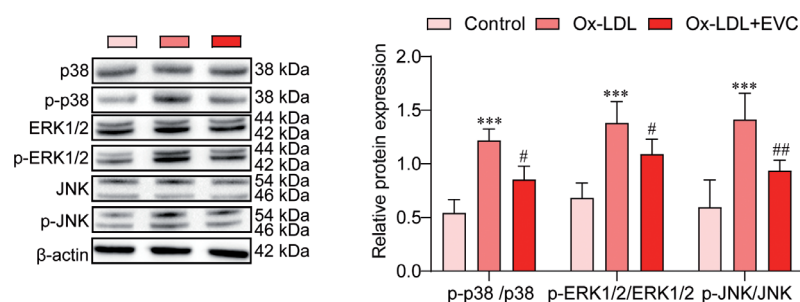


Figure 4. PCSK9 inhibitor Evolocumab exerts its effects by inhibiting the ROS/MAPK signaling pathway. Western blot detection of the expression levels of MAPK signaling pathway-related proteins p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK. *** $p < 0.001$ vs. Control. # $p < 0.05$, ## $p < 0.01$ vs. Ox-LDL.

of AS plaques was not observed in the mice in the control group. Vessels in the AS group presented typical AS plaques. These symptoms were all relieved after EVC treatment (Fig. 6B). The results of immunohistochemistry revealed that PCSK9 protein expression was upregulated in the AS group compared with the control group and that EVC treatment partially attenuated PCSK9 protein expression (Fig. 6C). The results of Western blot analysis revealed that, compared with those in the control group, the protein expression of Bax and cleaved-caspase-3 was upregulated and the protein expression of Bcl-2 was downregulated in the AS group, whereas, the treatment with EVC reversed the expression of these proteins (Fig. 6D). DHE staining revealed that ROS levels were significantly greater in the AS group than in the control group, whereas ROS levels were significantly lower in the AS+EVC group (Fig. 6E). In addition, compared with those in the control group, the SOD and GSH contents were lower and the MDA content was greater in the AS group, whereas the expression levels of the above oxidative stress-related indicators were reversed in the AS+EVC group (Fig. 6F).

Western blot analysis was performed to detect the phosphorylation levels of ERK1/2, JNK, and p38 proteins in the MAPK signaling pathway. The results showed that compared with the control group, the expression ratios of p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK were significantly increased in the AS group. In contrast, these phosphorylation levels were markedly reduced in the AS+EVC group (Fig. 6G). These results indicate that PCSK9 inhibitor EVC alleviates the progression of AS in mice.

Discussion

This study highlights the beneficial effects of the PCSK9 inhibitor EVC in attenuating the progression of AS through non-lipid metabolic pathways. Several key findings have been established: Firstly, EVC was shown to reduce blood lipid levels and ameliorate AS lesions in HFD-fed ApoE^{-/-} mice; secondly, it significantly inhibited Ox-LDL-induced apoptosis and oxidative stress in HUVECs; furthermore, this study demonstrated that the protective effects of EVC against Ox-LDL-induced endothelial injury are closely as-

sociated with the modulation of the ROS/MAPK signaling pathway. Collectively, these results indicate that EVC represents a promising therapeutic agent for the treatment of AS.

Endothelial cell injury is a common pathological basis of various CVD and is associated with high mortality and disability rates (Zhang et al. 2021). Ox-LDL-induced endothelial injury is considered a key factor in the pathogenesis and progression of AS (Bian et al. 2020). During the progression of AS, endothelial cell injury destroys the integrity and barrier function of endothelial cells, thereby promoting lipid deposition, plaque formation and thrombus formation (Zhang et al. 2015). Ox-LDL induces endothelial apoptosis and reduces the antioxidant capacity and nitric oxide synthesis of endothelial cells by altering the secretory activity of endothelial cells (Zhang et al. 2016). In addition, the expression of Bax, Bcl-2 and caspase-3 was abnormal in Ox-LDL-induced HUVECs, which was closely related to the Ox-LDL-induced endothelial cell damage response (Li et al. 2017). Consistent with previous findings, the results of this study demonstrate that Ox-LDL induction significantly reduced HUVECs cell viability and the levels of SOD and GSH, while increasing the apoptosis rate and the levels of ROS and MDA. Concurrently, it up-regulated the expression of Bax and cleaved-caspase 3 and down-regulated the expression of Bcl-2, collectively leading to HUVECs apoptosis and oxidative stress.

In addition, Ox-LDL induction also upregulated the expression of PCSK9. PCSK9 is expressed mainly in the liver, belongs to the proprotein convertase family and is involved in the progression of AS by regulating lipid metabolism and nonlipid metabolism pathways (Zeng et al. 2021). Previous studies have shown that PCSK9 is involved in endothelial apoptosis and that the knockout of PCSK9 can inhibit Ox-LDL-induced endothelial apoptosis and relieve the formation of atherosclerotic plaques (Xia et al. 2019). EVC is a selective PCSK9 inhibitor that is widely used in the clinical treatment of AS because of its ability to reduce blood lipids and improve vascular endothelial function (Safaeian et al. 2019; O'Donoghue et al. 2022). Previous studies have shown that EVC significantly reduces LDL-C levels and relieves AS through the inhibition of PCSK9 activity (Barale et al. 2021). In this study, animal experiments revealed that EVC could

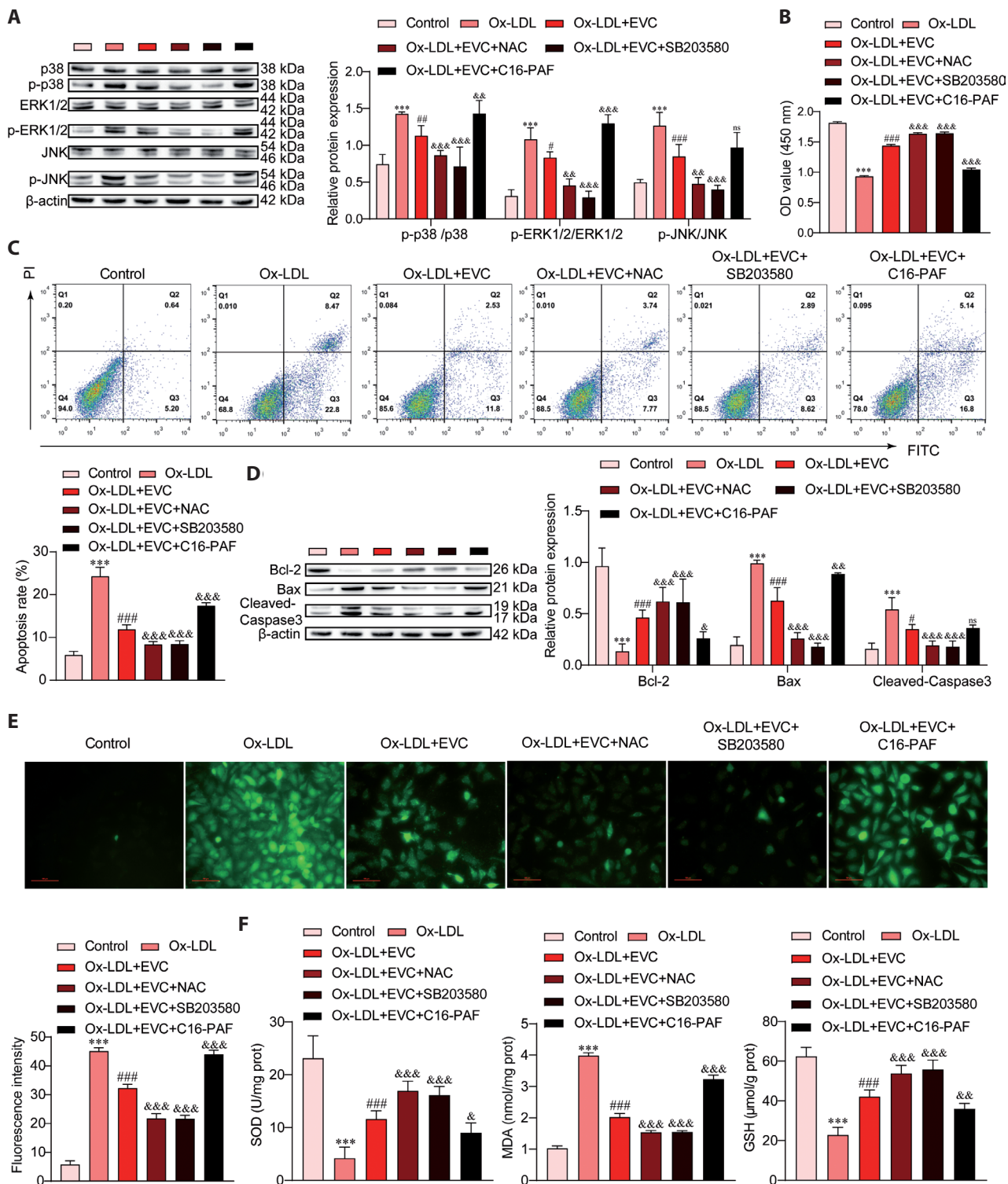


Figure 5. PCSK9 inhibitor Evolocumab suppresses Ox-LDL-induced apoptosis and oxidative stress in HUVECs by inhibiting the ROS/MAPK signaling pathway. **A.** Western blot detection of the expression levels of MAPK signaling pathway-related proteins p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK. **B.** Cell viability assessed by CCK-8. **C.** Apoptosis measured by flow cytometry. **D.** Western blot detection of the expression of Bcl-2, Bax, and cleaved-caspase-3. **E.** ROS content detected by DCFDA staining, scale bar: 100 μ m. **F.** SOD, MDA and GSH content of HUVECs analyzed by the kit. *** $p < 0.001$ vs. Control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Ox-LDL. & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ vs. Ox-LDL + EVC.

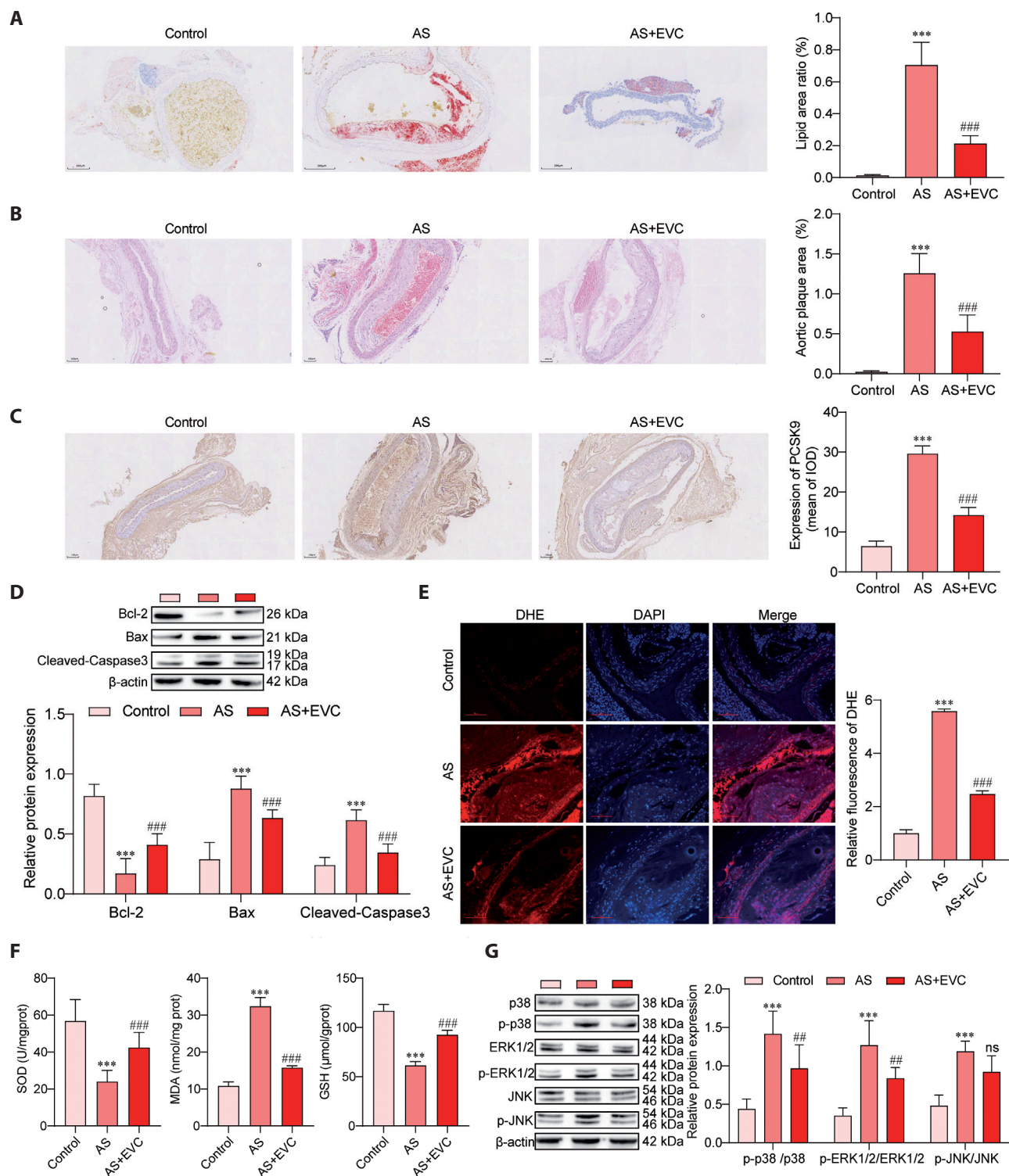


Figure 6. PCSK9 inhibitor Evolocumab alleviates the progression of AS in mice. **A.** Oil red O staining of aortic tissue, scale bar: 200 μ m. **B.** Pathological conditions of mouse aortic tissue analyzed by HE staining, scale bar: 100 μ m. **C.** PCSK9 expression in aortic tissue measured by immunohistochemistry, scale bar: 100 μ m. **D.** Expression of Bcl-2, Bax, and cleaved-caspase-3 measured by Western blot. **E.** ROS content analyzed by DHE staining, scale bar: 100 μ m. **F.** SOD, MDA, and GSH content in aortic tissue analyzed by the kit. **G.** Western blot detection of the expression levels of MAPK signaling pathway-related proteins p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK. *** $p < 0.001$ vs. Control. ## $p < 0.01$, ### $p < 0.001$ vs. AS.

reduce serum TG, TC and LDL-C levels while increasing serum HDL-C levels. In addition, EVC administration led to reduced aortic plaque formation and lipid droplet accumulation in the mice, thereby alleviating the progression of AS. EVC also has pleiotropic benefits, including anti-inflammatory and antioxidant activities, to help improve endothelial cell function (Safaeian et al. 2022; D'Onofrio et al. 2023). Research has shown a correlation between PCSK9 inhibition and apoptosis and oxidative stress in AS (Yurtseven et al. 2020). PCSK9 silencing can reverse the apoptotic effect of Ox-LDL and inhibit endothelial apoptosis (Wu et al. 2012). EVC protects HUVECs from H₂O₂-induced oxidative stress (Safaeian et al. 2022). In further investigating the protective effects of EVC against Ox-LDL-induced endothelial cell injury, we found that EVC attenuated the Ox-LDL-induced damage, resulting in increased cell viability, reduced apoptosis rate, accompanied by a significant decrease in ROS and MDA levels, and a marked increase in SOD activity and GSH content. This study demonstrates that the PCSK9 inhibitor EVC can suppress Ox-LDL-induced apoptosis and oxidative stress in HUVECs, thereby exerting a protective effect against Ox-LDL-induced endothelial injury.

During the AS process, the overproduction of ROS is considered the main response of cells to AS (Yang et al. 2017). Ox-LDL can increase the production of ROS in HUVECs, which can activate lipid peroxidation and lead to oxidative stress (Qin et al. 2015). There is increasing evidence that oxidative stress caused by excessive ROS is closely related to the progression of AS (Lu et al. 2020). For example, oxidative stress can lead to endothelial dysfunction, oxidative modification of LDL and atherosclerotic plaque formation (He et al. 2021). In addition, intracellular ROS generation can promote endothelial apoptosis (Wang et al. 2016). ROS can also activate the MAPK signaling pathway to increase the phosphorylation of p38, JNK and ERK (Xu et al. 2020). The MAPK signaling pathway is one of the most important pathways in the eukaryotic signal transduction network and plays a critical role in the regulation of endothelial cell survival, apoptosis, inflammation and dysfunction (Chen et al. 2021). The phosphorylation and activation of ERK1/2, JNK and p38MAPK have been reported to be involved in Ox-LDL-induced endothelial apoptosis (Chang H et al. 2018). The results of this study demonstrated that Ox-LDL significantly activated the phosphorylation of ERK1/2, JNK, and p38 in the MAPK signaling pathway, whereas treatment with the PCSK9 inhibitor effectively suppressed this activation, revealing a critical role of the ROS/MAPK pathway in the progression of AS. Further investigation showed that both NAC and SB203580 enhanced the protective effects of EVC against Ox-LDL-induced apoptosis and oxidative stress in HUVECs, while C16-PAF produced the opposite outcome. These findings indicate that blockade of the ROS/p38 MAPK signaling

pathway contributes to the protective mechanisms of EVC against Ox-LDL-mediated endothelial dysfunction.

In summary, the results of this study demonstrate that the PCSK9 inhibitor EVC significantly alleviates Ox-LDL-induced endothelial cell apoptosis and oxidative stress by inhibiting the ROS/MAPK signaling pathway, thereby attenuating the progression of AS. This finding holds important clinical translational value, as targeting the ROS/MAPK pathway not only provides new insights into the vascular protective mechanisms of PCSK9 inhibitors but also offers a potential therapeutic target for novel anti-AS strategies. Future drug interventions focusing on this pathway-whether used alone or in combination with existing lipid-lowering therapies-may provide more precise and effective treatment options for AS patients.

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Ethics approval. The Animal Experiment Ethics Review Committee of Kunming Medical University approved all the animal experiments (kmmu20221121). All methods were performed in accordance with the relevant guidelines and regulations and in accordance with the ARRIVE guidelines.

Data availability. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest. The authors declare that they have no competing interests.

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