Inflammation, Vol. 42, No. 2, April 2019 (© 2018)
DOI: 10.1007/s10753-018-0918-0

CrossMark

ORIGINAL ARTICLE

ApoM-S1P Modulates Ox-LDL-Induced Inflammation Through the PI3K/Akt Signaling Pathway in HUVECs

Zhi Zheng,[1] Yongzhi Zeng,[1] Xiao Zhu,[1] Ying Tan,[1] Yi Li,[1] Qian Li,[1] and Guanghui Yi[1,2]

Abstract—Studies have shown that apolipoprotein M (apoM), the main carrier of sphingosine-1-phosphate (S1P), is closely related to lipid metabolism and inflammation. While there are many studies on apoM and lipid metabolism, little is known about the role of apoM in inflammation. Atherosclerosis is a chronic inflammatory process. To clarify what role apoM plays in atherosclerosis, we used oxidized low-density lipoprotein (ox-LDL) to induce an inflammatory model of atherosclerosis. Our preliminary results indicate that ox-LDL upregulates the expression of S1P receptor 2 (S1PR2) in human umbilical vein endothelial cells (HUVECs). Ox-LDL-induced HUVECs were treated with apoM-bound S1P (apoM-S1P), free S1P or apoM, and apoM-S1P was found to significantly inhibit the expression of inflammatory factors and adhesion molecules. In addition, apoM-S1P inhibits ox-LDL-induced cellular inflammation *via* S1PR2. Moreover, apoM-S1P induces phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt, preventing nuclear translocation of nuclear factor-κB (NF-κB). PI3K-specific inhibitors and Akt inhibitors suppress apoM-S1P/S1PR2-induced interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) release and affect nuclear translocation of NF-κB. In conclusion, the results demonstrate for the first time that apoM-S1P inhibits ox-LDL-induced inflammation in HUVECs *via* the S1PR2-mediated PI3K/Akt signaling pathway. This finding may aid in the development of new treatments for atherosclerosis.

KEY WORDS: apoM-S1P; ox-LDL; inflammatory factors; adhesion molecules; atherosclerosis.

INTRODUCTION

Apolipoprotein M (apoM), one of the important components of high-density lipoproteins (HDL), has been explored because of its biological functions in diabetes, cardiometabolic risk, and lipid metabolism [1–3]. In addition to these biological functions, apoM is also closely related to the inflammatory process. Feingold et al. [4] reported that with the stimulation of lipopolysaccharide or zymosan, the level of apoM mRNA was significantly reduced in the liver, which may cause systemic inflammation. In addition, Ma et al. [5] showed that enhanced expression of apoM significantly mediates the anti-inflammatory effects of propofol in lipopolysaccharide-stimulated THP-1 macrophages. Hepatocytes are the main source of plasma apoM. Studies have shown that apoM is the main carrier of the membrane phospholipid messenger sphingosine-1-phosphate (S1P) [6]. Free S1P plays an important role in cell proliferation, differentiation,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-018-0918-0) contains supplementary material, which is available to authorized users.

[1] Institute of Cardiovascular Research, Key Laboratory for Atherosclerology of Hunan Province, University of South China, 28 W Changsheng Road, Hengyang, 421001, Hunan, China
[2] To whom correspondence should be addressed at Institute of Cardiovascular Research, Key Laboratory for Atherosclerology of Hunan Province, University of South China, 28 W Changsheng Road, Hengyang, 421001, Hunan, China. E-mail: ghyi6108@163.com

0360-3997/19/0200-0606/0 © 2018 Springer Science+Business Media, LLC, part of Springer Nature

lymphocyte migration, and pro-angiogenesis; however, less is known about the role of apoM-bound S1P (apoM-S1P). Recombinant apoM binds S1P with a KD of ~ 0.9 μM, indicating that apoM is the main carrier of membrane phospholipid messenger S1P [7, 8]. S1P bound to apoM accounts for approximately 65% of the total S1P in plasma and is thought to be a carrier that activates S1P receptors and promotes endothelial cell function. Approximately 30% of the S1P remaining in the plasma is bound to albumin [8]. As mentioned earlier, apoM-bound S1P and free S1P may play different roles in the chronic inflammatory response to atherosclerosis. Ox-LDL induces inflammation around the arterial wall, which is a key cause of atherosclerosis [9]. Therefore, we used ox-LDL to induce an *in vitro* model of inflammation. Our preliminary study confirmed that HUVECs mainly expressed S1PR1 and S1PR3, while ox-LDL significantly upregulated S1PR2 mRNA and protein levels in HUVECs. Next, our study showed that apoM-S1P inhibits the secretion of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in HUVECs under ox-LDL conditions, but this effect was eliminated by S1PR2 antagonists (JTE-013). In addition, apoM-S1P has the same effect on the adhesion molecules intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Moreover, apoM-S1P/S1PR2 induced the phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt and inhibited nuclear translocation of nuclear factor-κB (NF-κB). PI3K-specific inhibitors (LY294002) and Akt inhibitors (MK2206) blocked the anti-inflammatory effects of apoM-S1P on ox-LDL-induced HUVECs. Our results demonstrate for the first time that apoM-S1P inhibits ox-LDL-induced inflammation *via* the S1PR2-mediated PI3K/Akt signaling pathway, thereby protecting the vascular endothelium. This finding may provide us with more opportunities to treat chronic inflammatory diseases of atherosclerosis.

MATERIALS AND METHODS

Antibodies and Reagents

Dulbecco's modified Eagle's medium DMEM/high glucose was purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco, a branch of Thermo Fisher Scientific (Waltham, MA, USA). S1P was purchased from Cayman (Ann Arbor, MI, USA). ApoM (Human) recombinant protein (P01) was purchased from Abnova (Taiwan, China). Furthermore, S1PR1/3 antagonists (VPC23019) and S1PR2 antagonists (JTE-013) were obtained from Sigma-Aldrich (Saint Louis, Mo, USA). Akt inhibitor (MK2206) and PI3K inhibitor (LY294002) were acquired from Selleck Chemicals (Houston, TX, USA). Anti-IL-1β, anti-TNF-α, and anti-NF-κB p65 antibodies (rabbit polyclonal antibody) were obtained from Abcam (Cambridge, MA, USA). S1PR1, S1PR2, ICAM-1, VCAM-1, and β-actin antibodies (rabbit polyclonal antibody) were purchased from Proteintech (Chicago, IL, USA). An S1PR3 (rabbit polyclonal antibody) was obtained from Signalway Antibody (College Park, MA, USA). Human IL-1β, TNF-α, ICAM-1, and VCAM-1 ELISA kits were purchased from Neobioscience (Beijing, China). HRP-conjugated AffiniPure goat anti-rabbit lgG and CY3-conjugated AffiniPure goat anti-rabbit IgG were acquired from Proteintech (Chicago, IL, USA). All antibodies and reagents were commercially available and were of high quality.

Preparation of ApoM-Bound S1P

To load apoM with S1P, the necessary quantity of S1P was added to 1-μM human recombinant apoM and incubated for 1 h at room temperature. Then, the mixture was run through a desalting PD10 column (GE Healthcare, Beijing, China) with serum-free M200 medium to remove unbound S1P. Next, binding of apoM to S1P was verified by isoelectric focusing and intrinsic fluorescence quenching as described by Sevvana et al. [7]. The average S1P/apoM ratio was approximately 1.2. The prepared apoM-S1P was stored at − 20 °C.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HUVECs are repeated three times, and all produce similar results. Cells were grown in DMEM supplemented with 10% FBS (5% CO_2, 37 °C). HUVECs were used between passages 2 and 9. The cells were starved for 10 h in DMEM medium containing 1% FBS before treatment with the reagent. After pretreatment of HUVECs with ox-LDL for 12 h, the cells were washed three times with 3 mL of PBS and transferred to fresh medium for further culture. The cells are then treated with different reagents. Finally, HUVECs and culture media were harvested for RT-PCR, ELISA, Immunofluorescence, and Western blotting.

Blocking of S1P Receptors, PI3K, Akt

When HUVECs reached 70% confluence, the cells were starved for 10 h in DMEM containing 1% FBS. The HUVECs were then pretreated with ox-LDL for 12 h and transferred to fresh medium without ox-LDL. Next, the cells were pretreated with JTE-013, VPC23019, LY294002, or MK2206 for 30 min. Subsequently, apoM-S1P was added to the medium and cultured for 12 h. Finally, cells were collected for quantification of the different target proteins.

Real-time PCR and siRNA Transfection

Total RNA from HUVECs was extracted from PVAT by the Rnease Mini Kit (QIAGEN, Valencia, CA) and then reverse transcribed by qScript cDNA SuperMix (Quanta BioSciences, MD, USA). The cDNA was further synthesized by iScript cDNA Synthesis Kit (Bio-Rad, Shanghai, China). Finally, the cDNA was subjected to quantitative PCR (in triplicate) in a 7500 Fast Real-Time PCR system. β-Actin served as the control. RT-PCR primers were designed to amplify human β-actin, S1PR1, S1PR2, and S1PR3. The primers used are shown in Table 1.

When cells were 30% confluent, nontargeted control or targeted siRNAs (Delaware Ave, CA, USA) were transfected into HUVECs using DharmaFECT 3 Transfection Reagent (Thermo Fisher Scientific, MA, USA). The 24-well culture plates were incubated in a CO_2 incubator at 37 °C. The GFP reporter plasmid was transfected into HUVECs, and the transfection efficiency of siRNA was determined based on GFP fluorescence. The blocking effect of targeted siRNAs on HUVECs was monitored using a control siRNA targeting β-actin. In each case, cells were viewed at least three times, and representative photographs were taken.

Table 1. Primer sequences for RT-qPCR analysis are as follows:

Probe	Probe sequence
Human S1PR1	Sense 5′-GCACCAACCCCATCATTTAC-3′
	Antisense, 5′-TTGTCCCCTTCGTCTCTG-3′
Human S1PR2	Sense 5′-CAAGTTCCACTCGGCAATGT-3′
	Antisense 5′-CAGGAGGCTGAAGACAGAGG-3′
Human S1PR3	Sense 5′-TCAGGGAGGGCAGTATGTTC-3′
	Antisense 5′-GAGTAGAGGGGCAGGATGGT-3′
Human β-actin	Sense 5′-GAGACCTTCAACACCCCAG-3′
	Antisense 5′-TCAGGTCCCGGCCAGCCA-3′

Western Blot Analysis

Whole-cell protein was extracted using RIPA lyse buffer and PMSF (DLCS100, Beijing, China) at a 94:6 ratio to lysis HUVECs. HUVEC nuclear proteins (NF-κB p65) were extracted according to the instructions using the EpiQuik Nuclear Extraction Kit (AmyJet Scientific, Hubei, China). First, a BCA protein concentration kit (Solarbio, Beijing, China) was used to calculate the concentration of the protein to be tested. Proteins were then separated by SDS-PAGE (10-μg protein per lane, 12% gels), and transferred to PVDF membranes (Sigma-Aldrich, Saint Louis, Mo, USA). After blocking with 5% non-fat milk for 4 h, the membranes were incubated with the anti-β-actin antibody (or other antibodies) overnight, and the concentration is 1:1000. The next day, the membranes were incubated with a second antibody (1:5000) for 2 h with gentle shaking at room temperature. Finally, the target protein was visualized by eECL Western Blot Kit (eECL; Merck Millipore, Darmstadt, Germany), and each target protein was analyzed using ImageJ 1.38 (HIH, Bethesda, MD, USA) image processing software.

ELISA

HUVEC supernatants were harvested at the end of the treatment period. An equivalent amount of total protein (3 g) was loaded using a corresponding ELISA kit (Neobioscience, Beijing, China) according to the manufacturer's instructions to determine the levels of secreted IL-lβ (or TNF-α, ICAM-1, VCAM-1) in the culture medium.

Immunofluorescence

The HUVECs were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 (Solarbio, Beijing, China) in PBS for 30 min, and blocked with 10% BSA in PBS for 1 h at room temperature. HUVECs were stained with an NF-κB p65 monoclonal antibody at a dilution of 15 μg/mL in staining buffer overnight at room temperature, and then incubated with a Cy3-conjugated AffiniPure goat anti-rabbit lgG at a dilution of 1:200 for 1 h at room temperature. Next, HUVEC nuclei were counterstained for 30 min with 10 μg/mL DAPI solution (Solarbio, Beijing, China). The final images were captured on a fluorescence microscope (IX70; Olympus, Tokyo).

Statistical Analysis

Data were reported as the mean ± standard deviation. The values obtained were compared and analyzed using

GraphPad Prism 6.02 (San Diego, CA, USA). Difference analysis between groups was performed using Student's *t* tests. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Ox-LDL Significantly Upregulates S1PR2 mRNA and Protein Levels in HUVECs

Among the five S1P receptor subtypes, S1PR1-3 are expressed in many cell types, while S1PR4 is mainly expressed in immune cells and S1PR5 is expressed in the central nervous system [10]. During inflammation, the levels of S1P in different immune-related tissues and the expression of S1P receptors on the cell surfaces are modified, resulting in significant changes in the behavioral functions of the cells [11, 12].

To investigate the expression pattern of S1P receptors on HUVECs under ox-LDL conditions, we first performed real-time PCR analysis to identify the baseline expression pattern of S1P receptors on HUVECs. Real-time PCR analysis showed that HUVECs mainly expressed S1PR1 and S1PR3 (Fig. 1a). Western blot analysis also showed the same results (Fig. 1b). It has been reported that S1PR2 mRNA and protein levels in endothelial cells are significantly upregulated under inflammatory conditions [13]. Therefore, we verified whether the expression of S1PR2 in HUVECs changes under ox-LDL conditions. The cells were starved for 10 h in DMEM containing 1% FBS before treatment with the reagent. Then, they were treated with different concentrations (0, 25, 50, 75, and 100 μg/mL) of ox-LDL for 12 h. RT-PCR and Western blotting were performed to determine S1PR1, S1PR2, and S1PR3 mRNA and protein levels. The results indicated that as the ox-LDL concentration increased, the S1PR2 protein expression level also gradually increased and reached a maximum at 75 μg/mL, while the expression of S1PR1/S1PR3 protein remained unchanged (Fig. 2a, b). Next, ox-LDL (75 μg/mL) was incubated with HUVECs for different times (0, 1, 6, 12, and 24 h). The results indicated that ox-LDL upregulated the maximum expression of S1PR2 after 12 h (Fig. 2c, d). This suggests that the expression of S1PR2 in HUVECs changed significantly under ox-LDL conditions.

ApoM-S1P Inhibits the Ox-LDL-Induced Inflammation in HUVECs

To determine whether apoM-S1P produces anti-inflammatory effects on endothelial cells, we treated cells with free apoM, S1P, and apoM-S1P. HUVECs were first pretreated with ox-LDL for 12 h and then treated with apoM (1.0 μM), apoM-S1P (containing 1.0 μM S1P), or S1P (1.0 μM) for 24 h. Next, the protein levels and secretion levels of IL-1β, TNF-α, ICAM-1, and VCAM-1 were examined using the Western blot and ELISA, respectively. In addition, the secretion level of the anti-inflammatory factor IL-10 was examined. As shown in Fig. 3, compared to free apoM and S1P, apoM-S1P significantly inhibited ox-LDL-induced release of IL-1β, TNF-α, VCAM-1, and ICAM-1 and promoted the secretion of anti-inflammatory factor IL-10. We also performed a similar test with albumin and found that albumin and albumin-S1P had no significant effect on the HUVEC inflammatory response (see supplementary Fig. 1).

In addition, as shown in Fig. 4, apoM-S1P significantly inhibited the effect of ox-LDL-induced IL-1β and TNF-α release at 1 μM (12 h). The results with ICAM-1

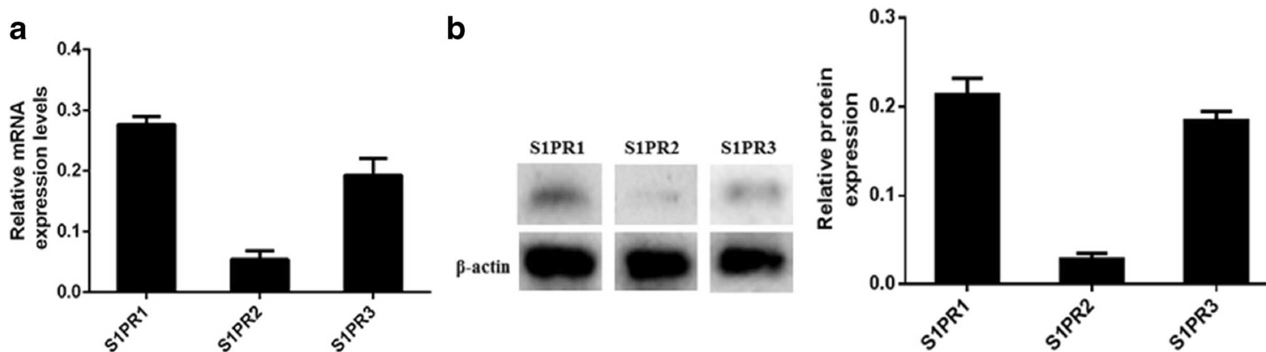


Fig. 1. The expression of S1P receptors in HUVECs under normal conditions. **a** Total RNA (100 ng) from HUVECs was amplified using primers for S1P receptors and β-actin. β-Actin was used as an internal standard. **b** Western blotting was used to detect the expression of S1P receptors in HUVECs.

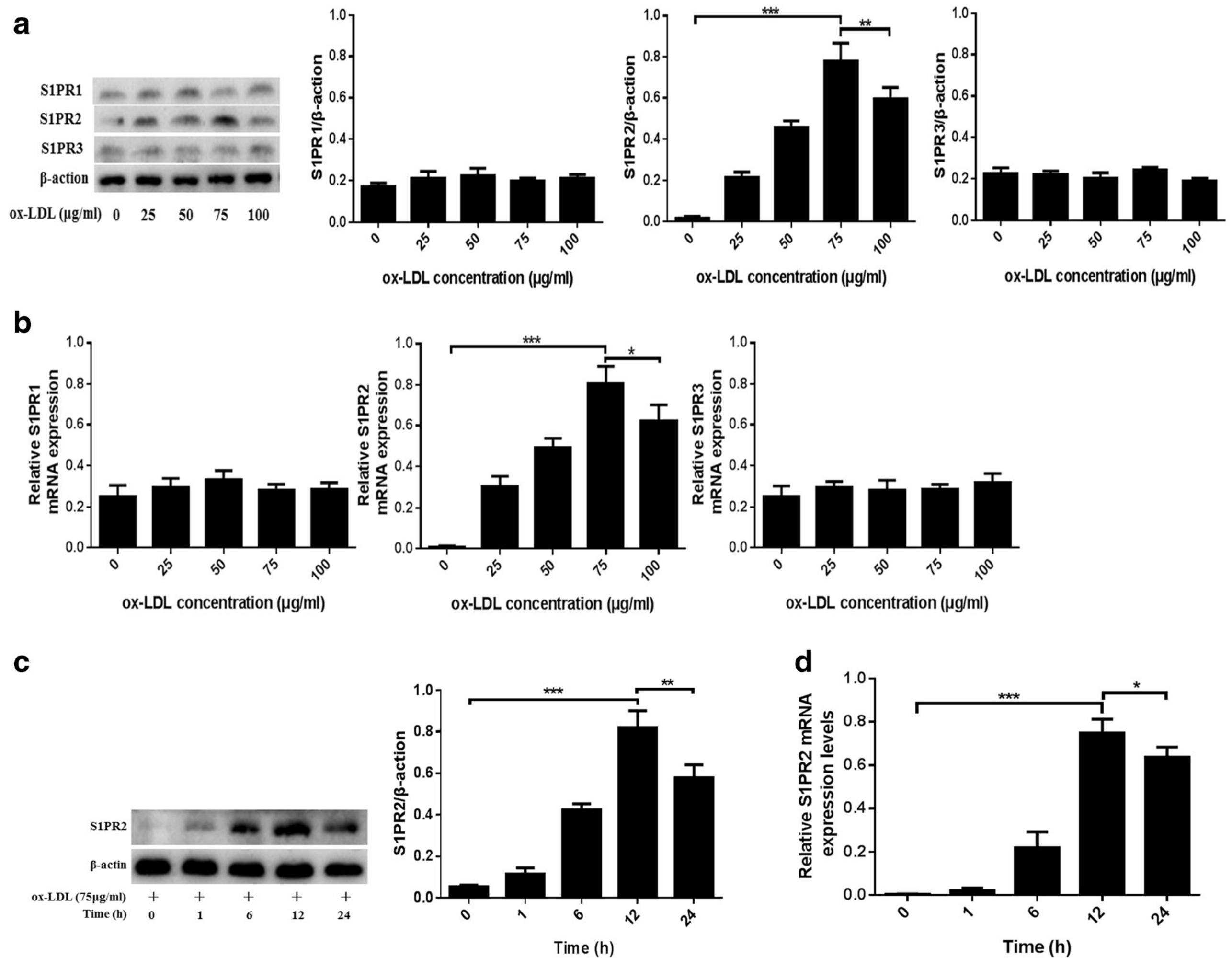


Fig. 2. Effect of ox-LDL on the expression of S1P receptors in HUVECs. **a** Western blotting was used to detect the protein expression of S1PR1, S1PR2, and S1PR3 in HUVECs at different concentrations of ox-LDL (0, 25, 50, 75, and 100 μg/mL). **b** RT-PCR was used to analyze the expression levels of S1PR1 mRNA, S1PR2 mRNA, and S1PR3 mRNA under various concentrations of ox-LDL. **c** HUVECs were cocultured with ox-LDL (75 μg/mL) for 0, 1, 6, 12, and 24 h, and the levels of S1PR2 were detected by Western blot. **d** RT-PCR was used to analyze the expression level of S1PR2 mRNA in HUVECs by ox-LDL at different times. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *n* = 5.

and VCAM-1 were also similar (see supplementary Fig. 2). Since free S1P and apoM had no significant effect on ox-LDL-induced inflammation of HUVECs, we used only apoM-S1P to explore its mechanism in cells in the following experiments.

ApoM-S1P Inhibits Ox-LDL-Induced Inflammation *via* S1PR2

Next, to determine whether apoM-S1P inhibits the release of inflammatory factors and adhesion molecules by targeting specific S1P receptors, HUVECs were pretreated with specific S1P receptor antagonists for 30 min before apoM-S1P stimulation. JTE-013 is a specific antagonist of S1PR2 (Ki = 0.69 mM), and VPC23019 is a specific antagonist of S1PR1 and S1PR3 (EC50 = 13 nM). Treatment of cells with JTE-013 significantly inhibited ox-LDL-induced IL-1β, TNF-α, ICAM-1, and VCAM-1 secretion, but VPC23019 had no significant effect (Fig. 5a, b). S1PR2 siRNA also inhibited ox-LDL-induced secretion of IL-1β and TNF-α by HUVECs (Fig. 5c, d). These results indicate that apoM-S1P inhibits ox-LDL-induced release of inflammatory factors and adhesion molecules in HUVECs *via* S1PR2.

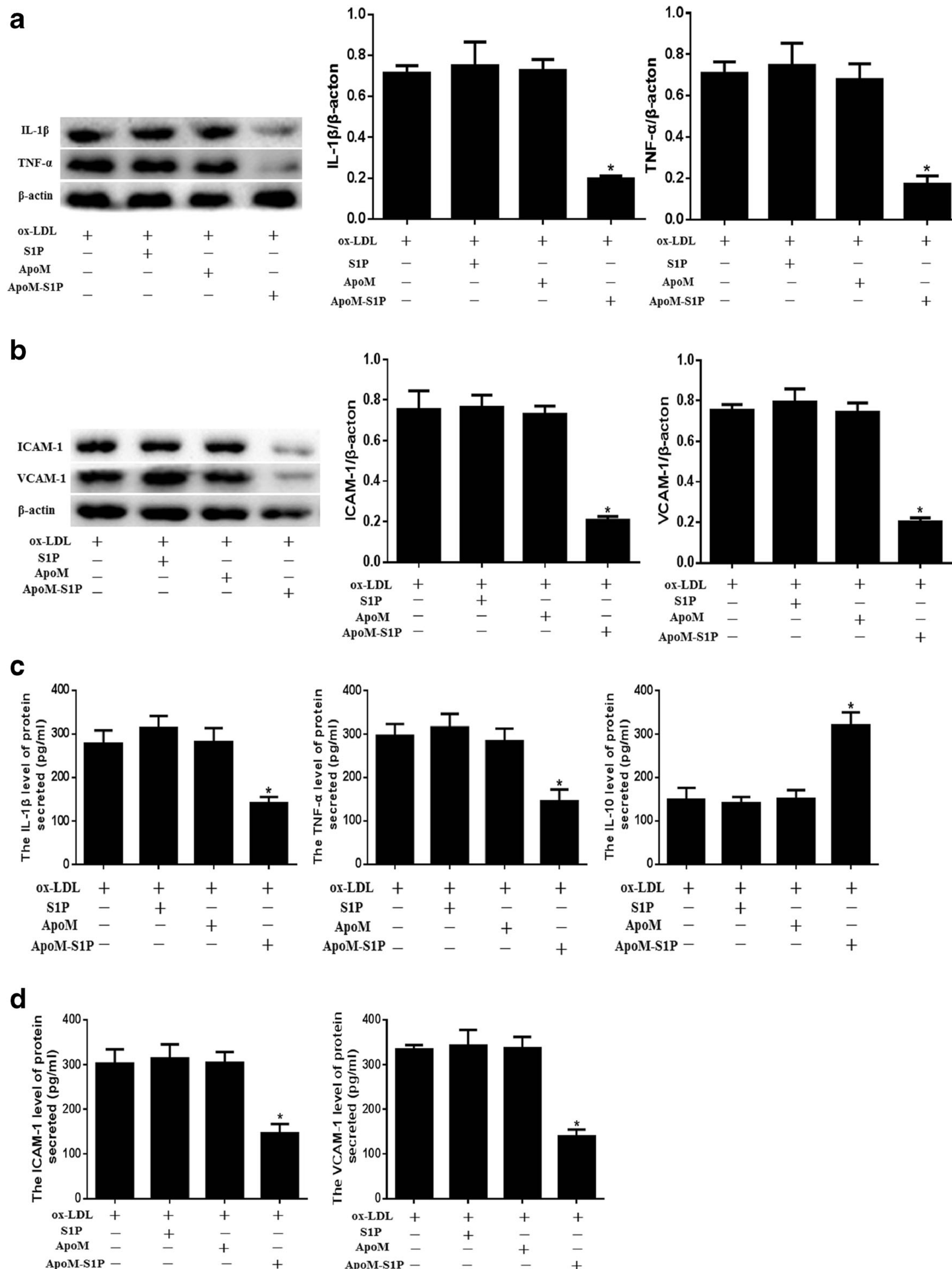


Fig. 3. Effect of apoM-S1P on inflammatory factors and adhesion factors induced by ox-LDL in HUVECs. **a**, **b** Western blotting was used to detect the expression of inflammatory factors and adhesion molecules (IL-1β, TNF-α, ICAM-1, and VCAM-1). **c**, **d** HUVECs were incubated with different treatment factors for 12 h; IL-1β, TNF-α, ICAM-1, and VCAM-1 secretion levels were then analyzed by ELISA. *$P < 0.05$, ox-LDL + apoM-S1P *vs.* ox-LDL, $n = 5$.

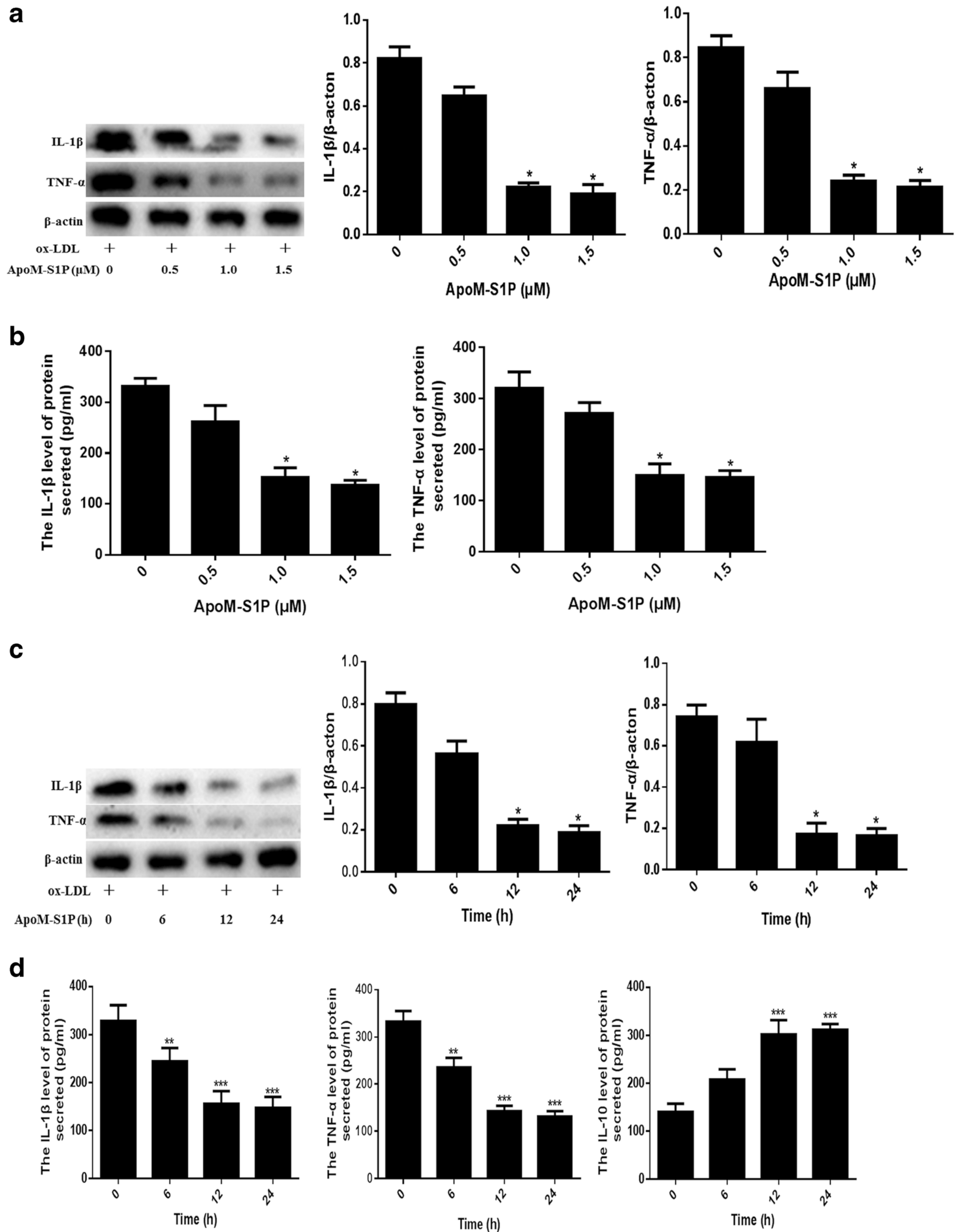


Fig. 4. Effect of different concentrations and times of apoM-S1P on ox-LDL-induced inflammation of HUVECs. **a**–**c** Western blotting was used to detect the release of IL-1β and TNF-α in ox-LDL-induced HUVECs by apoM-S1P at different concentrations and times. **b**–**d** ELISA was used to detect the secretion of IL-1β and TNF-α in ox-LDL-induced HUVECs by apoM-S1P at different concentrations and times. $^*P < 0.05$ *vs.* control group, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 5$.

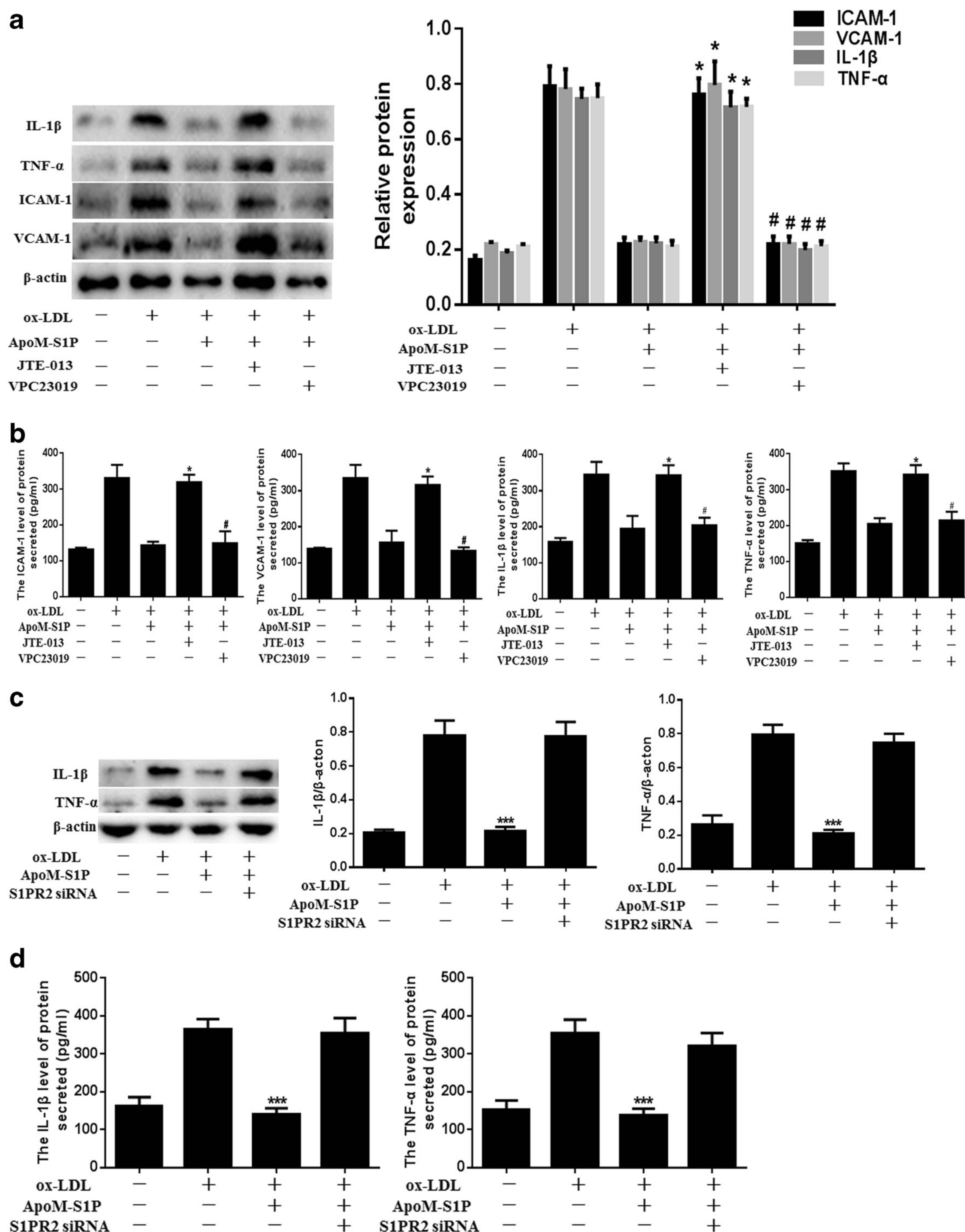
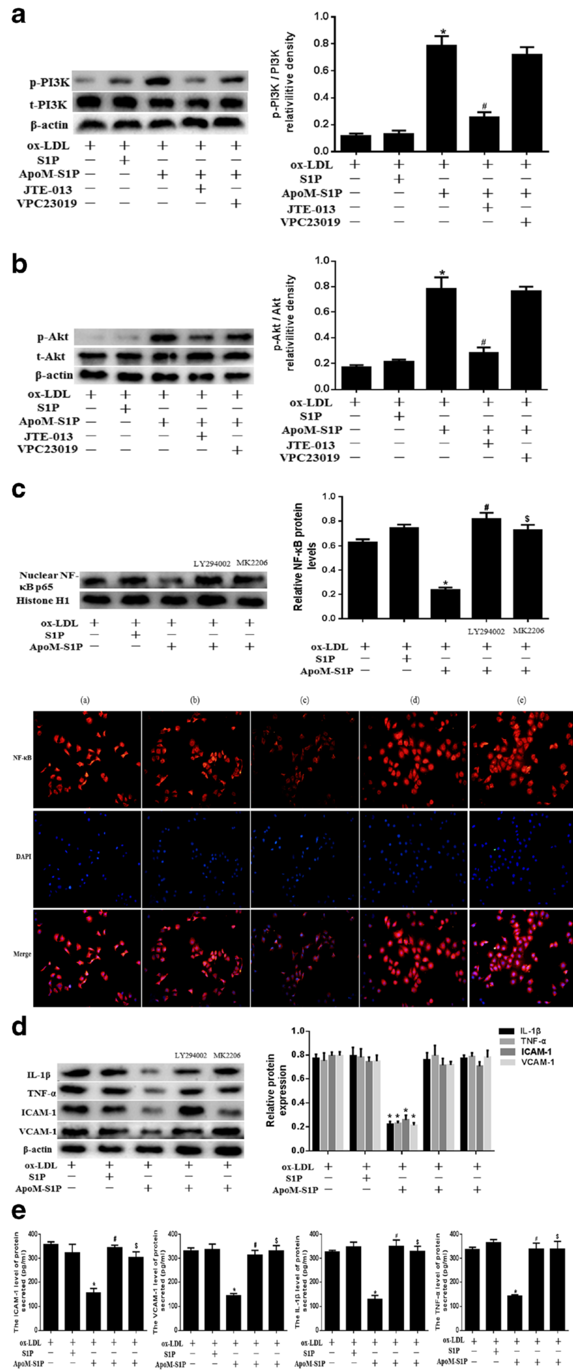


Fig. 5. ApoM-S1P inhibits ox-LDL-induced inflammation *via* S1PR2. **a**, **b** Cells were treated with JTE-013 (Ki = 0.69 mM) or VPC23019 (EC50 = 13 nM) and then stimulated with apoM-S1P for 12 h. The levels of IL-1β, TNF-α, ICAM-1, and VCAM-1 were detected by Western blotting and ELISA (*$P < 0.05$, ox-LDL + apoM-S1P *vs.* ox-LDL + apoM-S1P + JTE-013; #$P < 0.05$, ox-LDL + apoM-S1P + JTE-013 *vs.* ox-LDL + apoM-S1P + VPC23019; $n = 5$). **c**, **d** The cells were transfected with S1PR2 siRNA, and the expression of IL-1β and TNF-α were detected by Western blotting and ELISA (***$P < 0.001$, ox-LDL + apoM-S1P + S1PR2 siRNA *vs.* ox-LDL + apoM-S1P, $n = 3$).



◀ **Fig. 6.** Effect of PI3K/Akt pathway on inflammation-induced HUVECs treated with apoM-S1P. **a**, **b** Western blotting detected protein levels of t-PI3K, p-PI3K, t-Akt, and p-Akt (*$P < 0.05$, ox-LDL + apoM-S1P *vs.* ox-LDL; #$P < 0.05$, ox-LDL + apoM-S1P + JTE-013 *vs.* ox-LDL + apoM-S1P; $n = 5$). **c** Western blot and immunofluorescence analysis determined the level of NF-κB nuclear translocation. **a** Ox-LDL. **b** Ox-LDL + S1P. **c** Ox-LDL + apoM-S1P. **d** Ox-LDL + LY294002 + apoM-S1P. **e** ox-LDL + MK2206 + apoM-S1P. **d**, **e** HUVECs were treated with PI3K inhibitors or Akt inhibitors and then stimulated with apoM-S1P for 12 h. Western blotting and ELISA was used to detect the expression levels of IL-1β, TNF-α, ICAM-1, and VCAM-1 in the cells. Scale bar = 100 μm. Original magnification: × 20. *$P < 0.05$, ox-LDL + apoM-S1P *vs.* ox-LDL; #$P < 0.05$, ox-LDL + LY294002 + apoM-S1P *vs.* ox-LDL + apoM-S1P; \$$P < 0.05$, ox-LDL + MK2206 + apoM-S1P *vs.* ox-LDL + apoM-S1P; $n = 5$.

PI3K/Akt Is the Key to Mediating ApoM-S1P Inhibition of Ox-LDL-Induced Inflammation in HUVECs

It has been shown that PI3K/Akt is the downstream signaling pathway of S1P receptors [14], which play an important role in vascular inflammation [15, 16]. Additionally, NF-κB controls the expression of pro-inflammatory cytokines (such as IL-1, IL-6, and TNF-α) involved in inflammation. Therefore, we next evaluated whether PI3K/Akt is associated with apoM-S1P to during the inhibition of inflammatory responses in ox-LDL-induced HUVECs. HUVECs were pretreated with ox-LDL (75 μg/mL) for 12 h and then incubated with JTE-013, VPC23019, or other reagents. Western blot was used to detect t-PI3K, p-PI3K, t-Akt, and p-Akt protein levels. In addition, immunofluorescence was used to detect nuclear translocation of NF-κB. The results showed that apoM-S1P significantly increased the phosphorylation level of PI3K/Akt compared with that in the control group (Fig. 6a, b), while JTE-013 blocked the phosphorylation of PI3K/Akt in apoM-S1P-treated cells ($P < 0.05$). This result suggests that apoM-S1P increases p-PI3K and p-Akt protein levels, which are mediated through S1PR2. Moreover, apoM-S1P-induced nuclear translocation of NF-κB was alleviated by LY294002 and MK2206 (Fig. 6c, d).

To further verify whether the PI3K/Akt pathway is associated with inflammatory processes, we used a PI3K inhibitor (LY294002) and an Akt inhibitor (MK2206) to treat HUVECs and detected TNF-α and IL-1β secretion by Western blotting. The results showed that the release of TNF-α and IL-1β by apoM-S1P-treated cells was significantly reduced (Fig. 6d, e). In conclusion, these data suggest that apoM-S1P/S1PR2 inhibits the ox-LDL-induced secretion of IL-1β and TNF-α in HUVECs *via* the PI3K/Akt pathway (Fig. 7).

DISCUSSION

Studies have shown that vascular endothelial damage is the initial step in the development of atherosclerosis [17]. Ox-LDL is a major factor in early endothelial damage, monocyte adhesion, and foam cell formation [18]. Ox-LDL can help bind monocytes, neutrophils, and lymphocytes to endothelial cells by stimulating the expression of ICAM-1, VCAM-1, E-selectin, and P-selectin [14]. Endothelial cells also release cytokines such as IL-1β and TNF-α under pathological conditions, thereby causing a series of inflammatory reactions. At the same time, these inflammatory cytokines can also increase the expression of adhesion molecules on the surface of endothelial cells, promoting their adhesion with endothelium and subsequent injury to the endothelial cells. Our study showed that apoM-S1P could significantly downregulate the expression of inflammatory factors and adhesion molecules by inhibiting the inflammatory response of ox-LDL-induced HUVECs, thereby protecting endothelial cell function.

Obinata et al. [19] showed that S1P does not produce pro-inflammatory or anti-inflammatory effects on endothelial cells under steady-state conditions. However, excessive local concentrations of S1P can disturb the vascular S1P gradient, which leads to S1P playing a pro- or anti-inflammatory role in various types of cells. The expression pattern of the S1P receptor subtype on a given cell type, and the local S1P concentration seem to determine the effect of S1P. In our previous studies, free S1P produced a mild pro-inflammatory effect on endothelial cells but did not affect the study outcome [16]. Among these receptors, S1PR1-3 is expressed on HUVECs, while other receptors are not. Studies have shown that S1PR2 is upregulated in inflammation-induced endothelial cells [16, 20]. Therefore, we investigated the expression of S1P receptors in HUVECs under ox-LDL conditions. The results showed that ox-LDL significantly upregulated the expression of S1PR2 in HUVECs but did not affect the expression levels of S1PR1/S1PR3 (Fig. 2).

It is known that LDL particles can be oxidized, causing arterial lipid deposition and inflammation to promote cardiovascular disease. In contrast, HDL can reduce the oxidation of LDL, stimulate cholesterol outflow from macrophages, improve endothelial function, and further participate in related anti-inflammatory effects [21]. As one of the main components of HDL, apoM plays an important role in the function of HDL. Most S1P is synthesized intracellularly by sphingosine kinase (SPHK) and delivered to its corresponding S1P receptor (S1PR1-S1PR5) by apoM [22, 23]. S1P plays an essential role in cell proliferation, survival, angiogenesis, and immune cell trafficking through autocrine and paracrine signaling pathways [24]. However, there are few studies on apoM and inflammation. The human apoM gene was located in p21.31 on chromosome 6, which is a region of many genes associated with inflammation [1, 25]. Feingold et al. [4] showed that apoM is a negative acute response protein that decreases during inflammation, suggesting that apoM may be involved in the inflammatory response. In addition, Kurano et al. [26] treated mice with lipopolysaccharide and found that

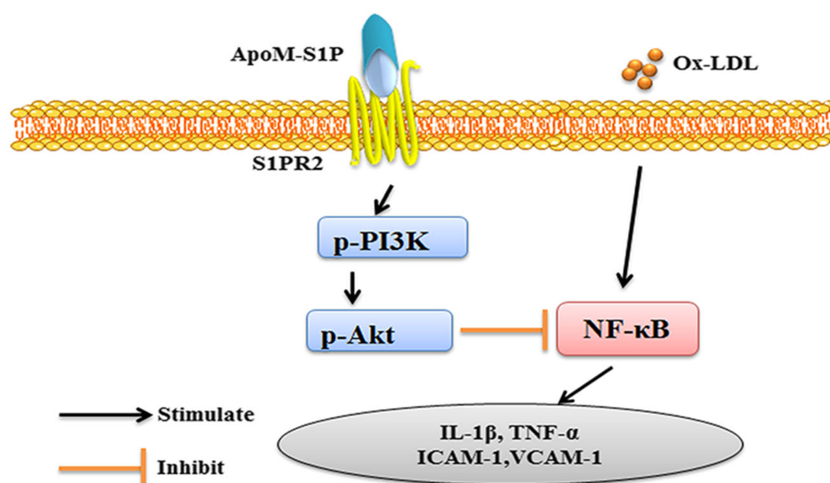


Fig. 7. Possible mechanism for ApoM-S1P mediating anti-inflammatory effects of HUVECs.

liver and kidney apoM expression and plasma apoM levels were suppressed. The overexpression of apoM increased survival in mice and regulated plasma creatinine and alanine aminotransferase levels, indicating that apoM has protective properties against lipopolysaccharide-induced organ injuries. Lipopolysaccharide and ox-LDL are both pro-inflammatory substances that can cause endothelial cell damage. However, whether apoM has protective properties against ox-LDL-induced endothelial damage has not been elucidated. In the present study, we found that apoM-S1P significantly inhibited the inflammatory effects of ox-LDL-induced HUVECs, whereas no such effects were found with free S1P and apoM (Fig. 3a, c). Previous studies have shown that TNF-α can significantly upregulate the expression of ICAM-1 and VCAM-1 [27, 28]. By contrast, in this study, we found that apoM-S1P could inhibit the expression of TNF-α in HUVECs. Therefore, we also examined the expression of ICAM-1 and VCAM-1 and found that their expression was also inhibited (Fig. 3b, d). In addition, we treated cells with S1P receptor antagonists and found that the S1PR2 antagonist but not the S1PR1/S1PR3 antagonist reduced the effect of apoM-S1P on IL-1β and TNF-α expression in inflammation-induced HUVECs (Fig. 5). This finding indicates that apoM-S1P inhibits inflammation of HUVECs primarily through S1PR2.

PI3K/Akt is involved in endothelial cell protection as a downstream signaling pathway of S1P [29]. NF-κB-mediated inflammation is also considered a key factor in the occurrence and development of atherosclerosis and other inflammatory diseases [30]. NF-κB is the downstream factor of cellular inflammation induced by ox-LDL. Moreover, NF-κB controls the expression of pro-inflammatory cytokines (such as IL-1, IL-6, and TNF-α), inducible enzymes, growth factors, and adhesion molecules involved in inflammation, apoptosis, and tumor cell proliferation [13]. Therefore, we next studied the effect of apoM-S1P on PI3K/Akt and found that apoM-S1P significantly enhanced PI3K/Akt activation, while S1PR2 antagonists blocked these effects (Fig. 6a, b). It was also shown that apoM-S1P/S1PR2 activates the PI3K/Akt pathway. Similarly, apoM-S1P inhibits NF-κB nuclear translocation induced by ox-LDL, which is abolished by PI3K/Akt inhibitors, as shown in Fig. 6c, d. Taken together, the data reveal a link between apoM, S1P, apoM-S1P, S1PR2, inflammatory factors, and endothelial protection, which enhances our understanding of apoM-S1P in atherosclerosis.

CONCLUSION

In summary, we preliminarily found that S1PR2 expression was upregulated in HUVECs under ox-LDL conditions and that apoM-S1P inhibited the secretion of IL-1β, TNF-α, ICAM-1, and VCAM-1 in ox-LDL-induced HUVECs. In addition, apoM-S1P inhibits ox-LDL-induced cellular inflammation *via* S1PR2. Overall, we found that the S1PR2/PI3K/Akt signaling pathway plays an important role in apoM-S1P inhibition of HUVEC inflammation.

FUNDING INFORMATION

The authors sincerely acknowledge the financial assistance provided by the National Natural Science Foundation of China (No. 81770490) and the Construct Program of the Key Discipline in Hunan Province (Basic Medicine Sciences in University of South China).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

REFERENCES

1. Borup, A., P.M. Christensen, and L.B. Nielsen. 2015. Apolipoprotein M in lipid metabolism and cardiometabolic diseases. *Current Opinion in Lipidology* 26: 48–55.
2. Dahlbäck, B. 2006. Apolipoprotein M–a novel player in high-density lipoprotein metabolism and atherosclerosis. *Current Opinion in Lipidology* 17: 291–295.
3. Nádró, B., L. Juhász, A. Szentpéteri, D. Páll, G. Paragh, and M. Harangi. 2018. The role of apolipoprotein M and sphingosine 1-phosphate axis in the prevention of atherosclerosis. *Orvosi Hetilap* 159: 168–175.
4. Feingold, K.R., J.K. Shigenaga, L.G. Chui, A. Moser, W. Khovidhunkit, and C. Grunfeld. 2007. Infection and inflammation decrease apolipoprotein M expression. *Atherosclerosis* 199: 19–26.
5. Ma, X., Y.W. Hu, Z.L. Zhao, L. Zheng, Y.R. Qiu, J.L. Huang, X.J. Wu, X.R. Mao, J. Yang, J.Y. Zhao, S.F. Li, M.N. Gu, and Q. Wang. 2013. Anti-inflammatory effects of propofol are mediated by apolipoprotein M in a hepatocyte nuclear factor-1a-dependent manner. *Archives of Biochemistry and Biophysics* 533: 1–10.
6. Christoffersen, C., M. Jauhiainen, M. Moser, B. Porse, C. Ehnholm, M. Boesl, B. Dahlbäck, and L.B. Nielsen. 2008. Effect of apolipoprotein M on high density lipoprotein metabolism and atherosclerosis in low density lipoprotein receptor knock-out mice. *J Biol Chem* 283: 1839–1847.

7. Sevvana, M., J. Ahnström, C. Egerer-Sieber, H.A. Lange, B. Dahlbäck, and Y.A. Muller. 2009. Serendipitous fatty acid binding reveals the structural determinants for ligand recognition in apolipoprotein M. *J Biol* 393: 920–936.
8. Christoffersen, C., H. binata, S.B. Kumaraswamy, S. Galvani, J. Ahnström, M. Sevvana, C. Egerer-Sieber, Y.A. Muller, and T. Hla. 2011. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. *Proceedings of the National Academy of Sciences of the United States of America* 108: 9613–9618.
9. Pirillo, A., and G.D. Norata. 2013. LOX-1, oxLDL, and atherosclerosis. *Mediators of Inflammation* 2013: 152786.
10. Vestri, A., F. Pierucci, A. Frati, L. Monaco, and E. Meacci. 2017. Sphingosine 1-phosphate receptors: Do they have a therapeutic potential in cardiac fibrosis? *Frontiers in Pharmacology* 8: 296.
11. Lai, W.Q., F.L. Chia, and B.P. Leung. 2012. Sphingosine kinase and sphingosine-1-phosphate receptors: Novel therapeutic targets of rheumatoid arthritis? *Future Medicinal Chemistry* 4: 727–733.
12. Liu, H., H. Jin, X. Yue, J. Han, P. Baum, D.R. Abendschein, and Z. Tu. 2017. PET study of sphingosine-1-phosphate receptor 1 expression in response to vascular inflammation in a rat model of carotid injury. *Molecular Imaging* 6: 1536012116689770.
13. Miraghazadeh, B., and M.C. Cook. 2018. Nuclear factor-kappaB in autoimmunity: Man and mouse. *Frontiers in Immunology* 9: 613.
14. Johnston, T.P. 2009. Poloxamer 407 increases soluble adhesion molecules, ICAM-1, VCAM-1 and E-selectin, in C57BL/6 mice. *The Journal of Pharmacy and Pharmacology* 61: 1681–1688.
15. Pyne, N.J., M. McNaughton, S. Boomkamp, N. MacRitchie, C. Evangelisti, A.M. Martelli, H.R. Jiang, S. Ubhi, and S. Pyne. 2016. Role of sphingosine 1-phosphate receptors, sphingosine kinases and sphingosine in cancer and inflammation. *Advances in Biological Regulation* 60: 151–159.
16. Ren, K., Y.J. Lu, Z.C. Mo, X. Liu, Z.L. Tang, Y. Jiang, X.S. Peng, L. Li, Q.H. Zhang, and G.H. Yi. 2017. ApoA-I/SR-BI modulates S1P/S1PR2-mediated inflammation through the PI3K/Akt signaling pathway in HUVECs. *Journal of Physiology and Biochemistry* 73: 287–296.
17. Hamed, S. 2006. Endothelial progenitor cells and atherosclerosis. *Harefuah* 145: 358–361.
18. Berliner, J.A., and J.W. Heinecke. 1996. The role of oxidized lipoproteins in atherosclerosis. *Free Radical Biology & Medicine* 20: 707–727.
19. Obinata, H. 2012. Sphingosine 1-phosphate in coagulation and inflammation. *Seminars in Immunopathology* 34: 73–91.
20. Du, J., C. Zeng, Q. Li, B. Chen, H. Liu, X. Huang, and Q. Huang. 2012. LPS and TNF-α induce expression of sphingosine-1-phosphate receptor-2 in human microvascular endothelial cells. *Pathology, Research and Practice* 208: 82–88.
21. Kosmas, C.E., I. Martinez, A. Sourlas, K.V. Bouza, F.N. Campos, V. Torres, P.D. Montan, and E. Guzman. 2018. High-density lipoprotein (HDL) functionality and its relevance to atherosclerotic cardiovascular disease. *Drugs Context* 7: 212525.
22. Hait, N.C., C.A. Oskeritzian, S.W. Paugh, S. Milstien, and S. Spiegel. 2006. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochimica et Biophysica Acta* 1758: 2016–2026.
23. Książek, M., M. Chacińska, A. Chabowski, and M. Baranowski. 2015. Sources, metabolism, and regulation of circulating sphingosine-1-phosphate. *Journal of Lipid Research* 56: 1271–1281.
24. Spiegel, S., and S. Milstien. 2003. Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nature Reviews. Molecular Cell Biology* 4: 397–407.
25. Luo, G., X. Zhang, and P. Nilsson-Ehle. 2004. Apolipoprotein M. *Lipids in Health and Disease* 3: 21.
26. Kurano, M., K. Tsuneyama, Y. Morimoto, T. Shimizu, M. Jona, H. Kassai, K. Nakao, A. Aiba, and Y. Yatomi. 2018. Apolipoprotein M protects lipopolysaccharide-treated mice from death and organ injury. *Thrombosis and Haemostasis* 118: 1021–1035.
27. Jiang, Y., L.L. Jiang, X.M. Maimaitirexiati, Y. Zhang, and L. Wu. 2015. Irbesartan attenuates TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression through suppression of NF-κB pathway in HUVECs. *European Review for Medical and Pharmacological Sciences* 19: 3295–3302.
28. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* 59: 1203–1211.
29. Takuwa, Y., Y. Okamoto, and K. Yoshioka. 2012. Sphingosine-1-phosphate signaling in physiology and diseases. *Biofactors* 38: 329–337.
30. Brown, J.D., C.Y. Lin, Q. Duan, G. Griffin, A. Federation, R.M. Paranal, S. Bair, G. Newton, A. Lichtman, A. Kung, T. Yang, and H. Wang. 2014. NF-kappaB directs dynamic super enhancer formation in inflammation and atherogenesis. *Molecular Cell* 56: 219–231.