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Adiponectin inhibits proliferation of vascular endothelial cells induced by Ox-LDL by promoting dephosphorylation of Caveolin-1 and depolymerization of eNOS and up-regulating release of NO

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ABSTRACT

Oxidized low density lipoprotein (ox-LDL) can induce the proliferation and differentiation of endothelial cells, which is one of the important mechanisms of ox-LDL atherosclerosis. Adiponectin is an endogenous bioactive polypeptide secreted by adipocytes, it participates in the metabolism of fat and glucose. It has the effect of reducing blood triglyceride and LDL content. Adiponectin also inhibits the abnormal proliferation and migration of endothelial cells, but its molecular mechanism is unclear. In this study, we used cell model of Ox-LDL-induced human aortic endothelial cells (HAECs) proliferation to analyze the molecular mechanism of APN inhibiting HAECs abnormal proliferation. The results showed that APN could inhibit the cell viability and DNA synthesis of HAECs after Ox-LDL treatment, up-regulate the apoptosis level and reduce the proportion of S + G2 phase cells. Further analysis showed that adiponectin could promote the dephosphorylation of Caveolin-1, which could dissociate eNOS and Caveolin-1, promote the phosphorylation of eNOS and enhance the synthesis of NO. NO increased expression levels of cleaved caspase 3 and p21 in the cells and inhibited the abnormal proliferation of HAECs. The regulation of phosphorylation and dephosphorylation of Caveolae-1 plays a key role in this process. Further study of the molecular mechanism of Caveolae-1 in the inhibition of HAECs abnormal proliferation by APN may reveal the potential of APN in the treatment of cardiovascular diseases.

1. Introduction

Atherosclerosis (AS) is a chronic vascular inflammatory disease, it was characterized by cholesterol and lipid accumulation which leading to lumen stenosis and sclerosis [1–3]. Abnormal proliferation of intimal endothelial cells (ECs) leads to intimal thickening of aorta, which plays an important role in the occurrence and development of AS [4–6]. Hyperlipidemia is one of the major risk factors for AS. Low density lipoprotein (LDL) can be oxidized to oxidized LDL (Ox-LDL) in the presence of a large number of oxygen free radicals in the body, ox-LDL can regulate the proliferation, apoptosis, migration and differentiation of human aortic endothelial cells (HAECs), and promote HAECs to form foam cells [7–9]. Therefore, the HAECs proliferation of artery intima induced by Ox-LDL is an important factor in atherosclerotic plaque formation.

Adiponectin (APN), a cytokine secreted by adipose tissue, is composed of 244 amino acids, also known as ACRP30 (adipocyte complement-related 30 kDa protein). It plays an important regulatory role in energy metabolism, anti-inflammatory and cardiovascular protection [10–13]. APN plays its biological role by binding to two G protein-coupled transmembrane receptors AdipoR1 and Adipo2, which are different in structure and function. APN was reported to play a protective role in cardiovascular diseases, it can inhibit the proliferation of HAECs by reducing the synthesis of cell DNA mediated by platelet-derived growth factors and fibroblast cytokines [14]. Plasma APN levels were significantly decreased in hypertensive patients, and hypoadiponectinemia was an independent risk factor for essential hypertension [15]. APN knockout mice are more prone to endothelial dysfunction [16]. APN can also play an anti-AS role by inhibiting the over-activation of NF-kB signaling pathway and the expression of adhesion molecules in vascular endothelial cells, and reducing the local adhesion of monocytes [17].

In the cardiovascular system, NO is mainly synthesized and secreted by HAECs through eNOS. NO can regulate the proliferation of HAECs by regulating the expression of cleaved caspase 3 and p21, and has the effects of anticoagulation, antioxidation and maintaining vasodilation [18–20]. Caveolae-1 is a class of lipid rafts that exist in endothelial cells, it is an important regulator of endothelial cell function. Caveolae-

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1 has a scaffold structure composed of 20 amino acids (82–101), which can bind to eNOS, inhibit its phosphorylation and reduce its function. When Caveolae-1 is dephosphorylated, it loses its ability to bind to eNOS, which increases the phosphorylation level of eNOS and promotes the production of NO [21]. Previous studies have shown that binding of APN with its receptor promotes the up-regulation of eNOS phosphorylation. APN could inhibit the production of oxygen free radicals (ROS) in endothelial cells induced by ox-LDL, and reverse the endothelial cell injury induced by TNF-α by inhibiting the signal pathway of NF-κB [22]. Whether APN can inhibit the abnormal proliferation of HAECs induced by ox-LDL and whether Caveolae-1 is involved in this process are still unclear.

In this study, we established a cell model of ox-LDL induced abnormal proliferation of HAECs, and explored the effects of APN on HAECs proliferation and its mechanism.

2. Materials and methods

2.1. Cell culture and siRNA transfection

Human aortic endothelial cells (HAECs) are purchased from American Type Culture Collection (ATCC). They were cultured with DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% Penicillin/Streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37 °C with 5% CO2. In ox-LDL treatment group, 50 μg/ml ox-LDL (Sigma-Aldrich, St. Louis, MO, USA) were added to the medium, 50 μg/ml APN (Sigma-Aldrich, St. Louis, MO, USA) were added to the medium of APN treated cells.

The siRNA transfection was performed using Lipofectamine 2000 kit (Life Technologies, Carlsbad, CA, USA) according to the manual protocol. The AdipoR1, AipoR2, Caveolae-1 and eNOS siRNA (Santa Cruz Biotechnology) were transfected into HAECs respectively.

2.2. Cell viability assay

The cell viability was detected with MTT Kit according to the kit's manual protocol. The cells in the logarithmic growth phase were digested with trypsin and inoculated into 96-well plates (5000 cell / well), they were cultured at 37 °C with serum free medium overnight. The medium was changed to contain serum and the cells were cultured with ox-LDL or APN, each well was added 10 μl MTT solution (0.5 mg/ml; Sigma-Aldrich; St. Louis, MO, USA) after culture for 0, 24 h and 48 h respectively and culture for 4 continually. After removing the medium, 200 μl DMSO (Sigma-Aldrich) was added into each well and incubated at room temperature for 10 min. The absorbance at 570 nm was detected by Microplate Reader (Multiskan MK3; Thermo Fisher Science, Inc.).

2.3. Cell cycle detection

HAECs were inoculated into 6-well plate (10,000/well) and cultured at 37 °C with serum free medium overnight. Then they were cultured in complete medium and medium containing ox-LDL and/or APN for 24 h. The cells were digested with trypsin and washed with pre cooled PBS for two times, they were fixed with 70% pre cooled ethanol at 4 °C overnight. The fixed solution was discarded and they were incubated with PBS containing 100 μg/ml RNase and 100 μg/ml PI at room temperature for 30 min. The cells were detected by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). CELLQUEST software (BD Biosciences) was used to analyze the ratio of G0/G1, S and G2/M in cells.

2.4. Cell apoptosis detected by flow cytometry assay

Apoptosis analysis was performed by AnnexinV-PI Analysis Kit (Beyotime, Shanghai, China) on FACScan (BD Biosciences, San Jose, CA, USA) according to manual. Cells in each group were collected, digested with trypsin without EDTA, and washed 3 times with ice-cold PBS. Cells were collected and resuspended with 195 μl Annexin V-FITC binding buffer. Add 5 μL Annexin V-FITC (Beyotime, Shanghai, China) according to the manufacturer's protocol. After 15 min of incubation at 4 °C refrigerator in the dark, 10 μl propidium iodide was added, and then incubates at room temperature for 10–20 min in the dark. The apoptosis rate was detected using flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA) and analyzed with CELLQUEST software (BD Biosciences). The experiment was repeated three times.

2.5. EdU incorporation assay

EdU can be inserted into the replicated DNA molecule. The increase in the proportion of EdU-positive cells represents a high proportion of cells with active DNA synthesis and a high proliferative capacity. In order to detect DNA synthesis in different groups of cells, the cells were inoculated into 2-well plate (2×10^4/well) and cultured at 37 °C with serum free medium overnight. Then they were cultured in complete medium containing ox-LDL and/or APN and 20 μM Edu for 24 h. They were washed twice with pre-cooled PBS, and fixed with pre-cooled 4% paraformaldehyde for 20 min. They were stained with Cell-LightTM EdU In Vitro Imaging Kit (Beyotime Biotechnology Co., Ltd., Shanghai, China) after treatment with 0.5% Triton X-100. The nucleus was stained with DAPI (Beyotime Biotechnology Co., Ltd., Shanghai, China) and the EdU positive cells were observed by inverted immunofluorescence microscopy.

2.6. NO detection

Cell culture supernatants were collected from different treatment groups, the NO content was determined within 30 min using Total Nitric Oxide Assay Kit (Griess method, Beyotime Biotechnology Co., Ltd., Shanghai, China) according to the instructions. At the same time, RIPA lysates containing cocktail protease inhibitors (Roche; Basel, Switzerland) were used to lyse cells, the protein content was detected by Bradford protein assay kit (Bio-Rad; Hercules, CA, USA). NO level was normalized by protein content of cultured cells in different groups, and the ratio of nitrite and nitrate (n mol) to protein content (μg) was calculated.

2.7. RNA extraction and qRT-PCR

Total RNA was extracted using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and purity were detected using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 1 μg RNA was subjected to reverse transcription using a MMLV Reverse Transcriptase Kit (TAKARA Biotechnology (Dalian) CO., Ltd., Dalian, China). qPCR was performed using a PCR amplification kit (TAKARA Biotechnology (Dalian) CO., Ltd., Dalian, China) on ABI StepOne Plus system (Applied Biosystems, Waltham, MA). The quantification method used was the $2^{-\Delta\Delta CT}$ method. The thermocycling conditions were as follows: Pre-degeneration at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s, GAPDH gene was used as an internal control. The primers used in this study are shown in Table 1.

Table 1
Primers used in this study.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
eNOS	ATCCGAGACATGATTGGACCG	TCCACAATGGCTTGAACTTGG
caveolin-1	ACAAGCCCAACAACAAGG	TTCCAAATGCCGTCAAAA
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

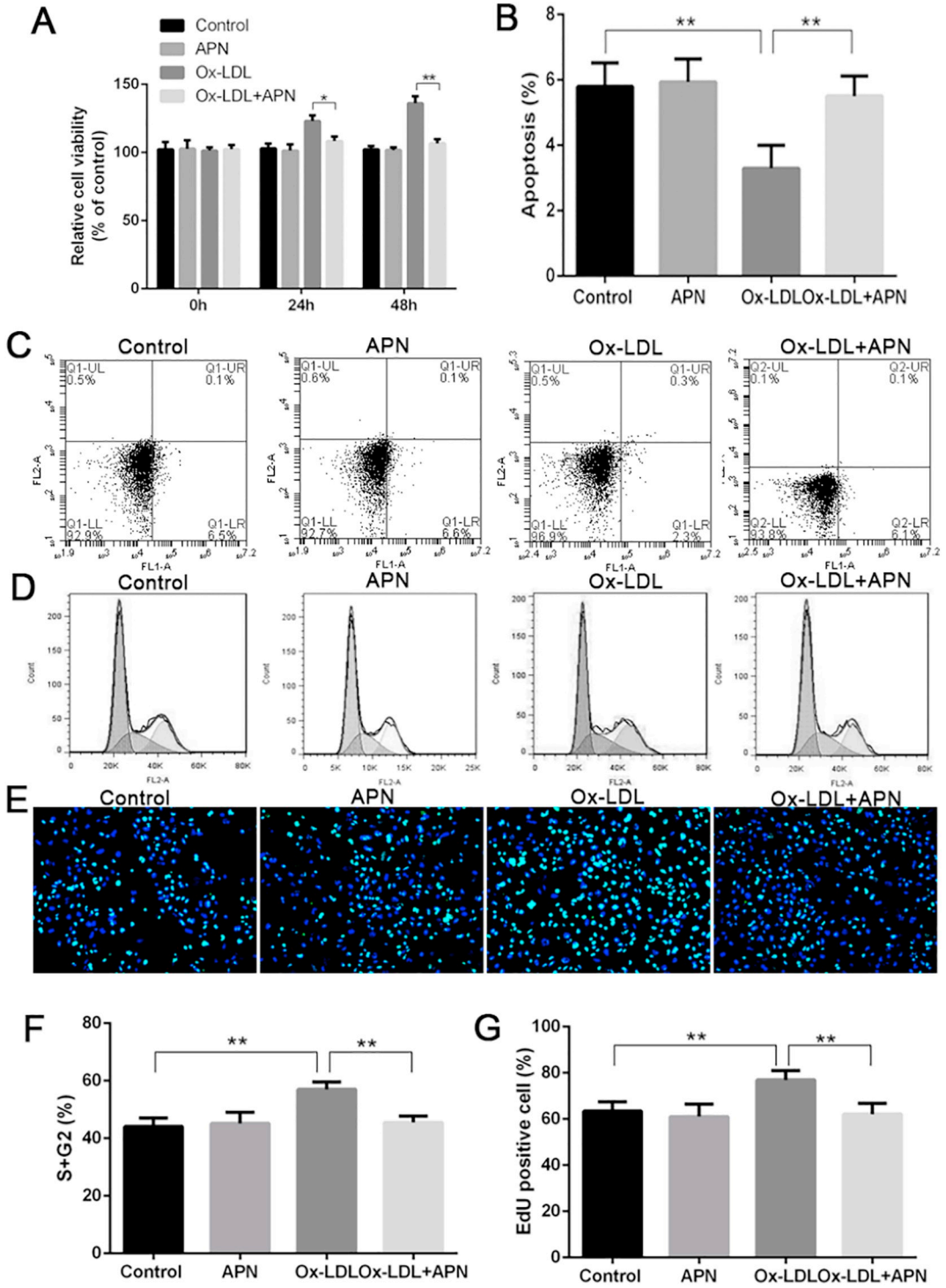

Fig. 1. APN could inhibit the proliferation of HAECs induced by ox-LDL
A: MTT results showed that APN could inhibit the increase of HAECs activity induced by Ox-LDL; B, C: Apoptosis detection showed that Ox-LDL could down-regulate the apoptosis rate of HAECs, while APN could bring it back to its normal level; D, E: Ox-LDL could significantly increase the proportion of S + G2 phase cells of HAECs, APN treatment could reduce the proportion of S + G2 phase cells to the same level as that of the control group; F, G: Ox-LDL treatment could upregulate the proportion of EdU positive cells in HAECs, while APN could bring it back to its normal level.
$^{*}P < 0.05$; $^{**}P < 0.01$.

2.8. Western blotting method

Cells in the logarithmic growth period were harvested and split with Cell Lysis Solution (Sigma-Aldrich, St. Louis, MO, USA) according to instructions. The protein concentration was determined using BCA kit (Beyotime, Shanghai, China) according to instructions. Proteins (50 μg per lane) were separated using 12% SDS-PAGE. Proteins were then electrotransferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The PVDF membrane was rinsed with TBS for 10–15 min, placed in TBS/T blocking buffer containing 5% (*w/v*) skimmed milk powder. It was incubated at room temperature for 2 h following the addition of an appropriate dilution of primary antibodies

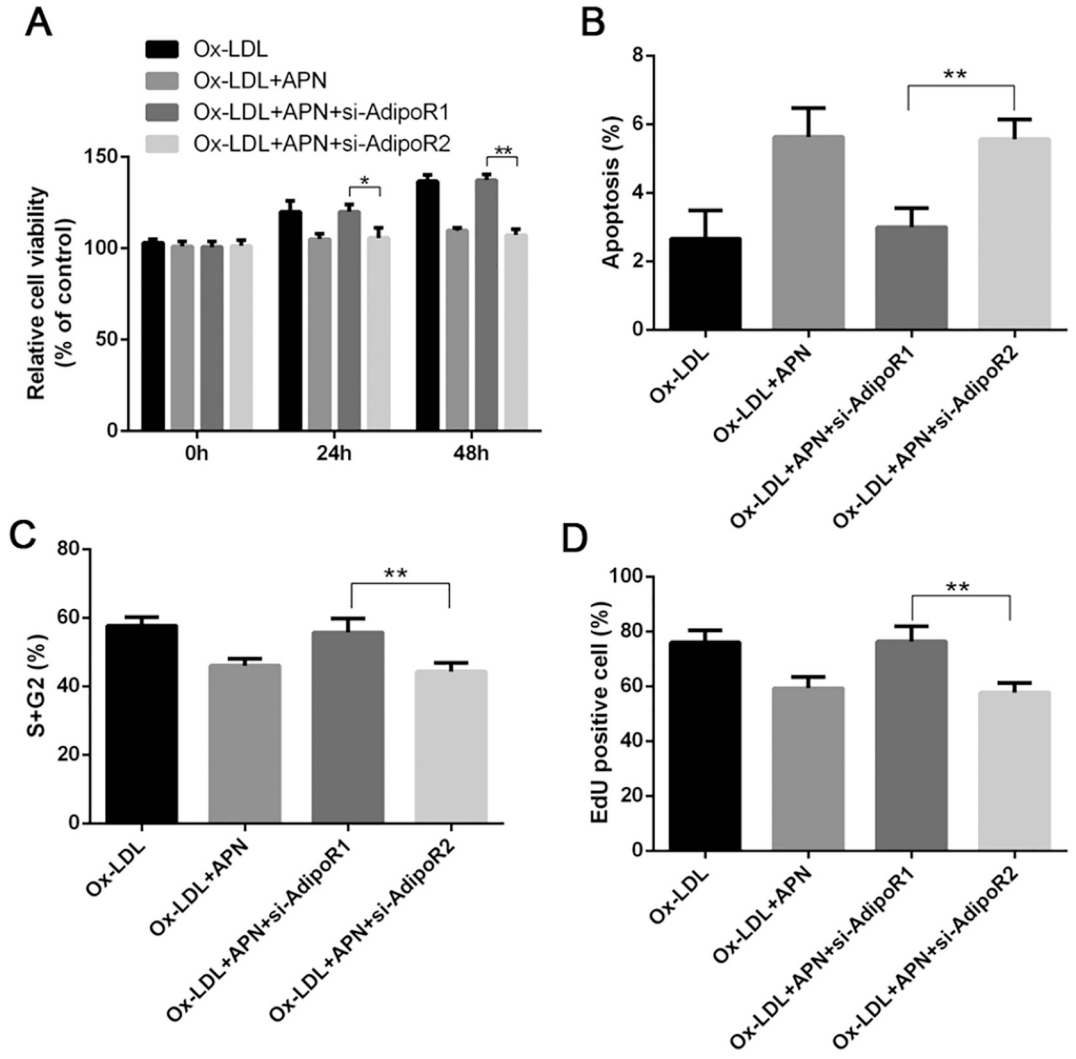

Fig. 2. APN inhibited HAECs proliferation induced by Ox-LDL dependent on AdipoR1 receptor
A: The ability of APN to inhibit the viability of HAECs cells decreased after down-regulation of AdipoR1, but down-regulation of AdipoR2 had no significant effect on the viability of HAECs; B: The ability of APN to promote HAECs apoptosis decreased after downregulation of AdipoR1, while the down-regulation of AdipoR2 had no significant effect on HAECs apoptosis; C: APN lost its ability to inhibit the increase of S + G2 phase cell proportion after down-regulation of AdipoR1, but down-regulation of AdipoR2 had no significant effect on it; D: APN lost the ability to inhibit the increase of the EdU-positive cell proportion after down-regulation of AdipoR1, but down-regulation of AdipoR2 had no significant effect on it.
$^{**}P < 0.01$.

rabbit anti-caveolin-1 (BD Biosciences, San Diego, CA), mouse anti-p-caveolin-1(Tyr-14) (BD Biosciences, San Diego, CA) , rabbit anti-eNOS (Cell Signaling Technology, Danvers, MA), rabbit anti-p-eNOS(Ser-1177) (Cell Signaling Technology, Danvers, MA) and anti-β-actin (BD Biosciences, San Diego, CA) respectively. They were incubated at 4 °C overnight. The membrane was then rinsed with TBST three times (5–10 min/wash) and then incubated at room temperature for 1 h with horseradish peroxidase-labeled secondary antibody (1:50,000; Abcam, Cambridge, UK; diluted with TBST containing 0.05% (w/v) skimmed milk powder). The membrane was then rinsed three times with TBST (5–10 min/wash). Protein bands were detected using an enhanced chemiluminescence kit (Perkin-Elmer Inc.) and quantified as the ratio to β-actin. Quantification was performed using ImageQuant LAS 4000 (GE Healthcare, Japan).

2.9. Co-immunoprecipitation assay

The proteins were extracted from the cells of different groups with NETN buffer containing cocktail protease inhibitor (Roche; Basel, Switzerland) after they were treated for 48 h. The protein content was detected by Bradford protein assay kit (Bio-Rad; Hercules, CA, USA). Immunocoprecipitation of 1 mg total protein with 2 μg eNOS antibody or non-specific IgG antibody (BD Biosciences, San Diego, CA) was performed. eNOS and its associated proteins were pulled down using IgA coated magnetic Dynabeads (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The pulled-down proteins were collected and boiled for 5 min, they were detected by SDS-PAGE and Western blotting method.

2.10. Statistical analysis

The data were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All results are presented as the mean ± standard deviation (SD). Student's *t*-test and one-way ANOVA test were used to evaluate the differences among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

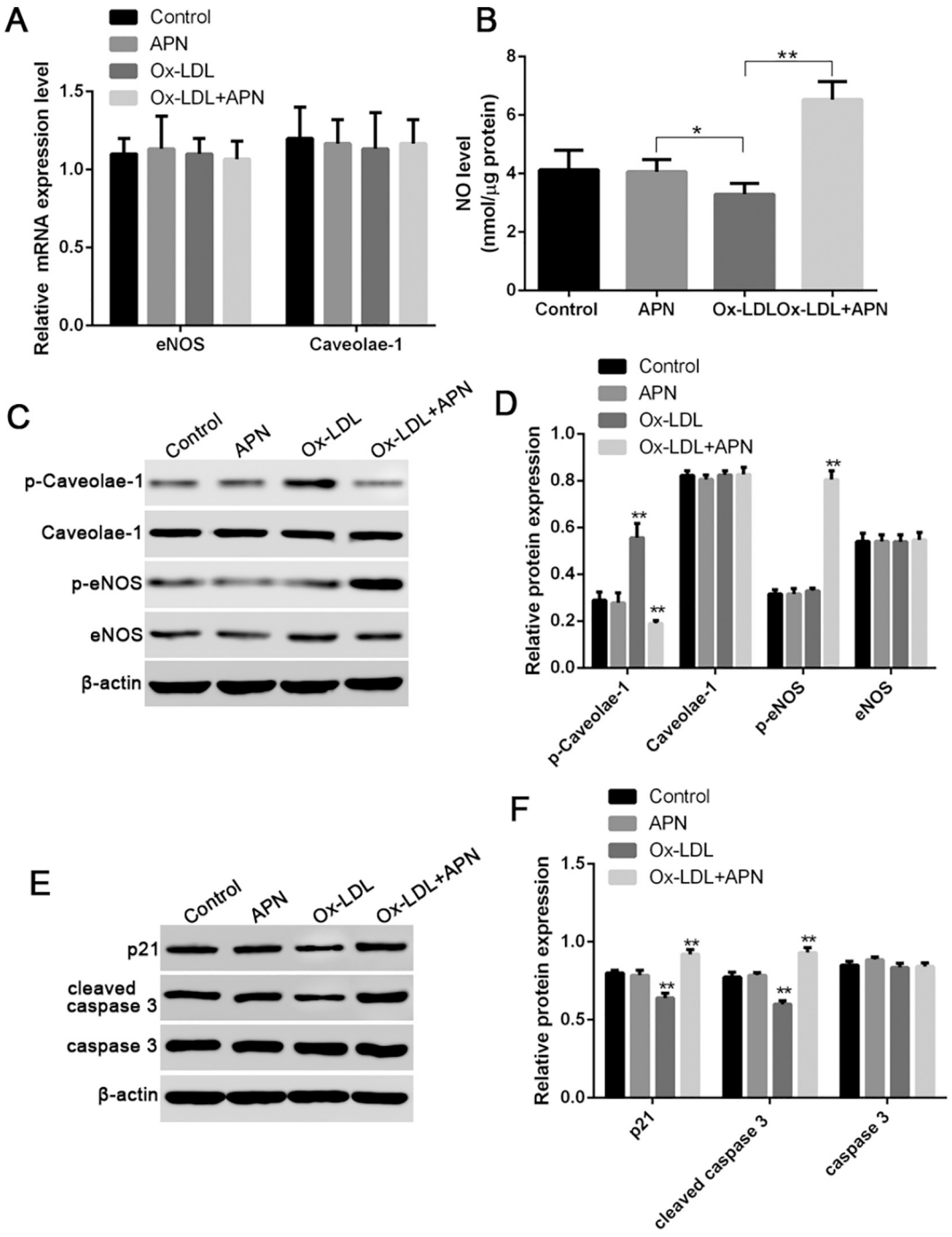

Fig. 3. APN promoted the release of NO and regulated HAECs proliferation induced by ox-LDL through increasing the phosphorylation level of eNOS
A: RT-PCR results showed that ox-LDL and APN had no effect on the mRNA expression levels of Caveolae-1 and eNOS; B: Ox-LDL could inhibit the secretion of NO in HAECs, APN could reverse this process, but APN did not affect the secretion of NO in normal cells. C, D: Western blotting results showed that ox-LDL and APN had no effect on the protein levels of Caveolae-1 and eNOS. Ox-LDL could promote the phosphorylation of Caveolae-1, and did not affect the phosphorylation level of eNOS. APN could inhibit Caveolae-1 phosphorylation induced by ox-LDL and promote eNOS phosphorylation. E, F: APN could reverse the decreased expression of apoptosis related proteins cleaved caspase 3 and p21 in HAECs induced by ox-LDL.
$^{*}P < 0.05$; $^{**}P < 0.01$.

3. Results

3.1 APN could inhibit the proliferation of HAECs cells induced by ox-LDL and promote apoptosis.

The results of MTT detection showed that the cell viability of HAECs was enhanced after they were treated with ox-LDL with time dependencies. Apoptosis and cell cycle analysis also showed that ox-LDL could inhibit the apoptosis of HAECs and up-regulate the proportion of S + G2 phase cells. The number of EdU positive cells increased in ox-LDL treated HAECs. These results indicated that ox-LDL could promote HAECs proliferation and inhibit apoptosis. When APN was added, the proliferation of HAECs induced by ox-LDL was inhibited, the proportion of S + G2 phase cells decreased, the number of EdU positive cells decreased, and the apoptosis level increased. In addition, there was no significant difference in the proliferation and apoptosis of HAECs cells treated with APN alone compared with the control group, which

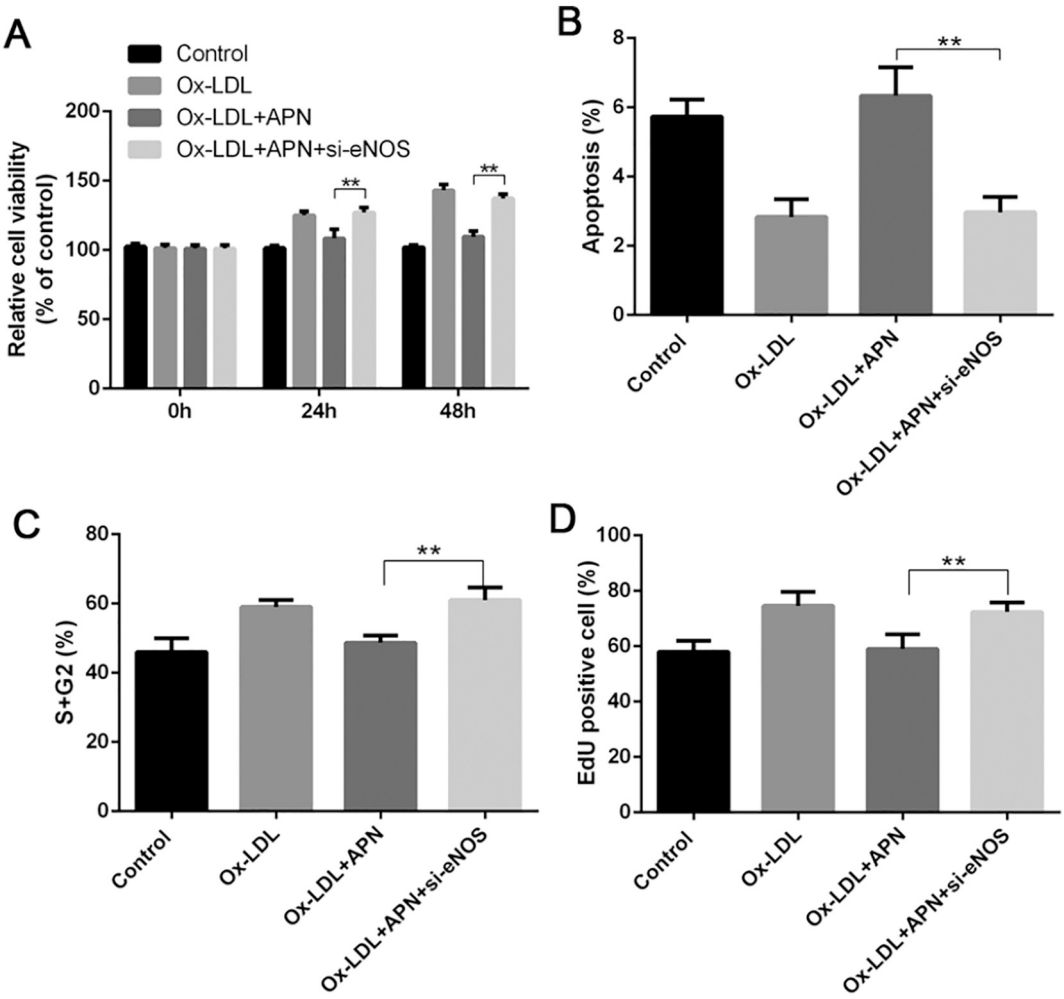

Fig. 4. After down regulating the expression of eNOS, APN could not inhibit the proliferation of HAECs induced by ox-LDL
A: After siRNA was used to down-regulate the expression of eNOS, the inhibitory effect of APN on the proliferation of HAECs cells induced by ox-LDL disappeared; B: APN could not inhibit the enhanced anti apoptotic effect of HAECs induced by ox-LDL after siRNA was used to down-regulate the expression of eNOS; C: The inhibitory effect of APN on increasing S + G2 phase ratio in HAECs induced by ox-LDL disappeared after siRNA was used to down-regulate the expression of eNOS; D: The inhibitory effect of APN on increasing EdU positive cell proportion of HAECs induced by ox-LDL also disappeared.
**P < 0.01.

suggesting that APN itself had no significant effect on the proliferation and apoptosis of HAECs cells at the concentration used in this study (Fig. 1).

3.2 APN could inhibit proliferation of HAECs cells induced by ox-LDL through AdipoR1.

To determine the role of APN receptors in this process, HAECs were transfected with siRNA targeting AdipoR1 and AdipoR2 respectively. It was found that the effect of APN on HAECs proliferation and apoptosis disappeared completely after down-regulation of AdipoR1, while down-regulation of AdipoR2 did not affect the effect of APN on HAECs proliferation and apoptosis. This indicated that the inhibition of APN on ox-LDL induced proliferation of HAECs cells was mediated through receptor AdipoR1 (Fig. 2).

3.1. APN could promote the phosphorylation of eNOS and the secretion of NO in HAECs cells induced by Ox-LDL, and could induce the up-regulation of the expression of pro-apoptotic molecules

RT-PCR results showed that ox-LDL and APN had no effect on the mRNA expression levels of Caveolae-1 and eNOS. Western blotting results also showed that ox-LDL and APN had no effect on the protein levels of Caveolae-1 and eNOS. Ox-LDL could up-regulate the p-Caveolae-1level and did not affect the p-eNOS level, but APN could inhibit Caveolae-1 phosphorylation induced by ox-LDL and promote eNOS phosphorylation. It was found that ox-LDL could inhibit the secretion of NO in HAECs, while APN could up regulate NO secretion. Ox-LDL could inhibit the expression levels of cleaved caspase 3 and p21 in HAECs, and the expression levels of cleaved caspase 3 and p21 increased after APN treatment (Fig. 3). These results suggested that APN may promote eNOS phosphorylation and NO expression by inhibiting Caveolae-1 phosphorylation. NO further increased the expression levels of cleaved caspase 3 and p21 in the cells and inhibited the abnormal proliferation of HAECs.

3.2. APN could inhibit the proliferation of HAECs cells induced by ox-LDL relying on NO secretion mediated by eNOS

After siRNA was used to down-regulate the expression of eNOS, the inhibitory effect of APN on the proliferation of HAECs induced by ox-LDL disappeared. APN could not increase the expression levels of eNOS, p-eNOS and NO secretion. The inhibitory effect of APN on increasing S + G2 phase ratio in HAECs induced by ox-LDL disappeared. The inhibitory effect of APN on increasing EdU positive cell proportion of HAECs induced by ox-LDL also disappeared (Fig. 4).

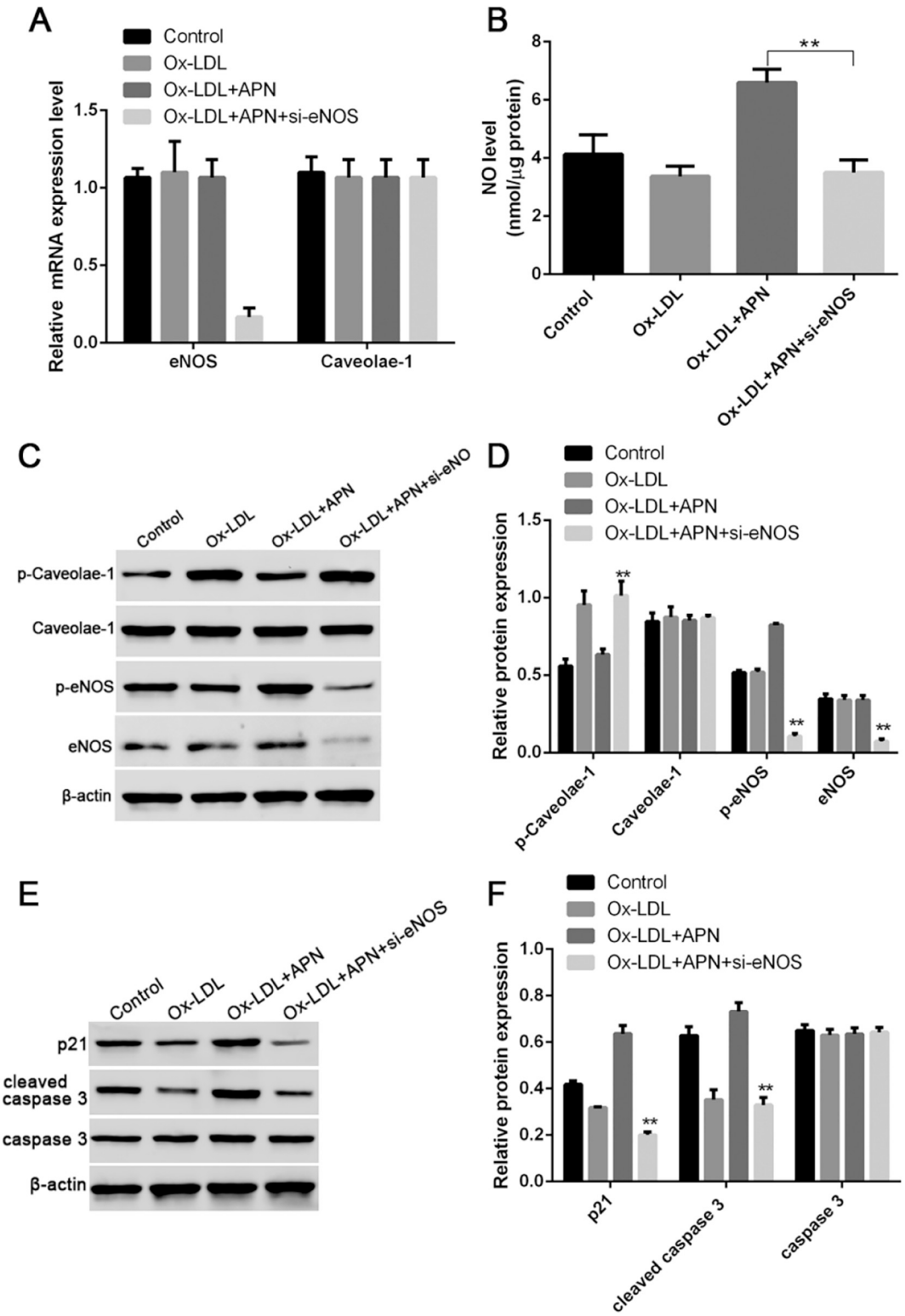

Fig. 5. Down regulation of eNOS expression could inhibit NO secretion induced by APN and the expression of apoptosis related proteins
A: Down regulation of eNOS had no effect on the expression level of Caveolae-1 mRNA; B: Down regulation of eNOS expression could inhibit NO secretion induced by APN; C, D: Down regulation of eNOS could decrease the expression levels of eNOS and p-eNOS, and APN could not inhibit the phosphorylation of Caveolae-1; E, F: The expression levels of cleaved caspase 3 and p21 could not be up regulated by APN after siRNA was used to down-regulate the expression of eNOS
**P $<$ 0.01.

APN had no effect on the expression level of Caveolae-1 mRNA and protein, but it could inhibit the phosphorylation of Caveolae-1. NO secretion induced by APN could be inhibited and the expression levels of cleaved caspase 3 and p21 could not be up regulated after siRNA was used to down-regulate the expression of eNOS (Fig. 5). These results indicated that APN inhibited the abnormal proliferation of HAECs cells induced by ox-LDL depending on NO release induced by eNOS.

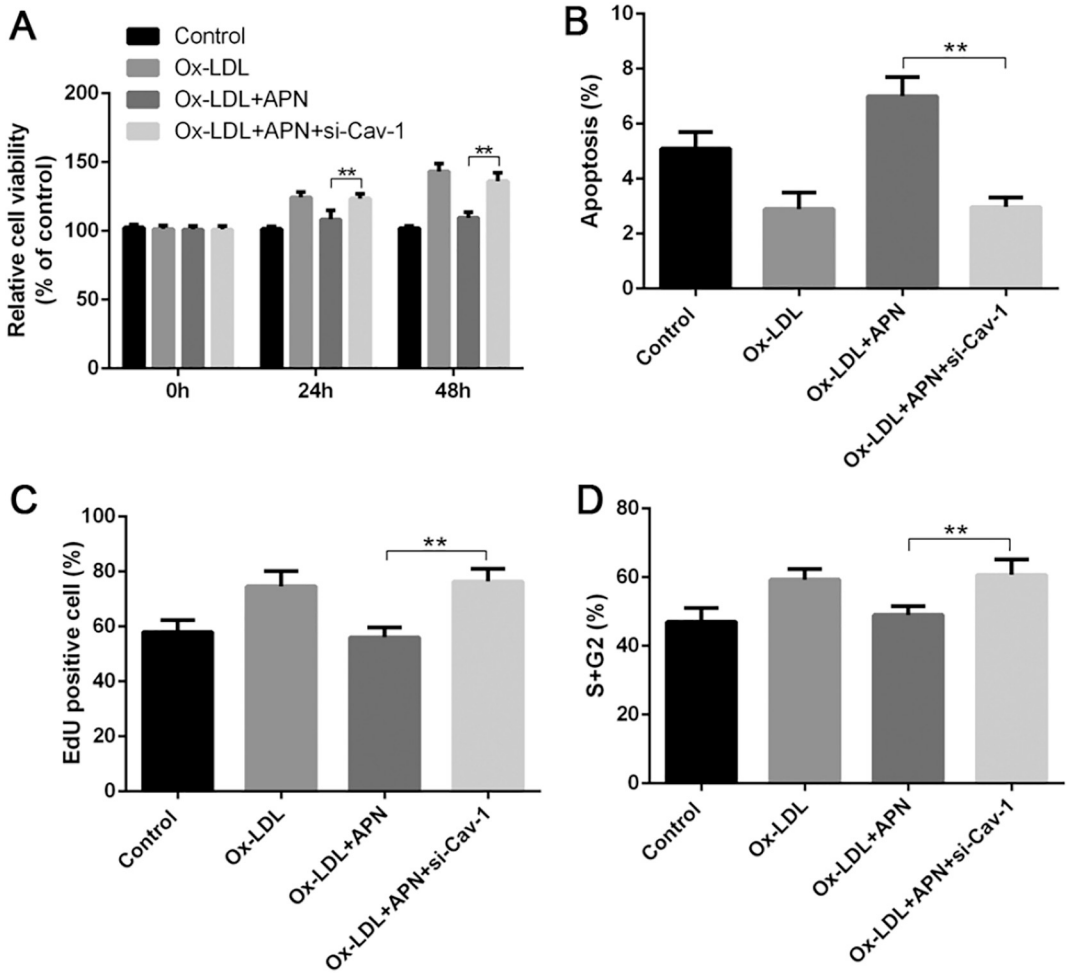

Fig. 6. The inhibitory effect of APN on the proliferation of HAECs cells induced by ox-LDL disappeared after down-regulating the expression of Caveolae-1
A: The inhibitory effect of APN on the enhanced cell viability of HAECs induced by ox-LDL disappeared after inhibiting the Caveolae-1 expression by siRNA; B: The inhibitory effect of APN on the enhanced anti apoptosis of HAECs induced by ox-LDL disappeared after inhibiting the Caveolae-1 expression by siRNA; C: APN could not inhibit the increased proportion of EdU positive cells in HAECs after inhibiting the Caveolae-1 expression by siRNA; D: APN could not inhibit the increased S + G2 phase ratio in HAECs induced by ox-LDL after inhibiting the Caveolae-1 expression by siRNA.

3.3. APN could inhibit the proliferation of HAECS cells induced by ox-LDL depending on the existence of Caveolae-1

Down regulation of eNOS expression could affect the secretion of downstream NO, but did not affect the expression and phosphorylation of Caveolae-1, which suggesting that Caveolae-1 may be the upstream regulator of eNOS. To clarify the role of Caveolae-1, we used siRNA to down-regulate the expression of Caveolae-1, and found that the inhibitory effect of APN on the proliferation of HAECs cells induced by ox-LDL disappeared. The eNOS mRNA expression level was not affected. Western blotting results showed that the expression levels of eNOS and p-eNOS and NO secretion level were not up-regulated treated by APN after down regulation of Caveolae-1 expression. The protein levels of cleaved caspase 3 and p21 were also not up-regulated treated by APN after down regulation of Caveolae-1 expression. These results suggested that Caveolae-1 played a key role in the process of APN-induced NO secretion and inhibiting the abnormal proliferation of HAECs cells induced by ox-LDL (Figs. 6, 7).

3.4. APN could reduce eNOS combined with p-Caveolae-1 by reducing p-Caveolae-1 level

Co-immunoprecipitation results showed that eNOS had strong binding force with p-Caveolae-1, but almost had no combination with Caveolae-1. Ox-LDL could promote the p-Caveolae-1 expression in HAECs cells, while APN could inhibit the p-Caveolae-1 level. The down regulation of p-Caveolae-1 could promote the phosphorylation of eNOS and promote the secretion of NO (Fig. 8).

4. Discussion

Atherosclerosis is a chronic vascular inflammatory disease characterized by atherosclerotic plaques consisted of foam cells, leukocytes, platelets, inflammatory smooth muscle cells and endothelial cells [22]. Ox-LDL is an important factor causing vascular injury and contributing to the pathogenesis of AS. In the early stage of AS, ox-LDL enters the subendothelial layer of vascular endothelial cells by injuring vascular endothelial cells, triggering a series of pathological events [23]. Many studies have shown that ox-LDL could stimulate the proliferation of HAECs cells [9,22,23]. Therefore, the proliferation of HAECSs induced by Ox-LDL is thought to be closely related to the occurrence and development of AS. Inhibition of HAECs proliferation is an important strategy for the prevention and treatment of AS. In this study, we found that APN at physiological level could inhibit the abnormal proliferation of HAECs induced by ox-LDL.

APN has two isomeric receptors, AdipoR1 and AdipoR2, which are very similar in structure and are membrane proteins with seven transmembrane domains. Both AdipoR1 and AdipoR2 existed in aortic

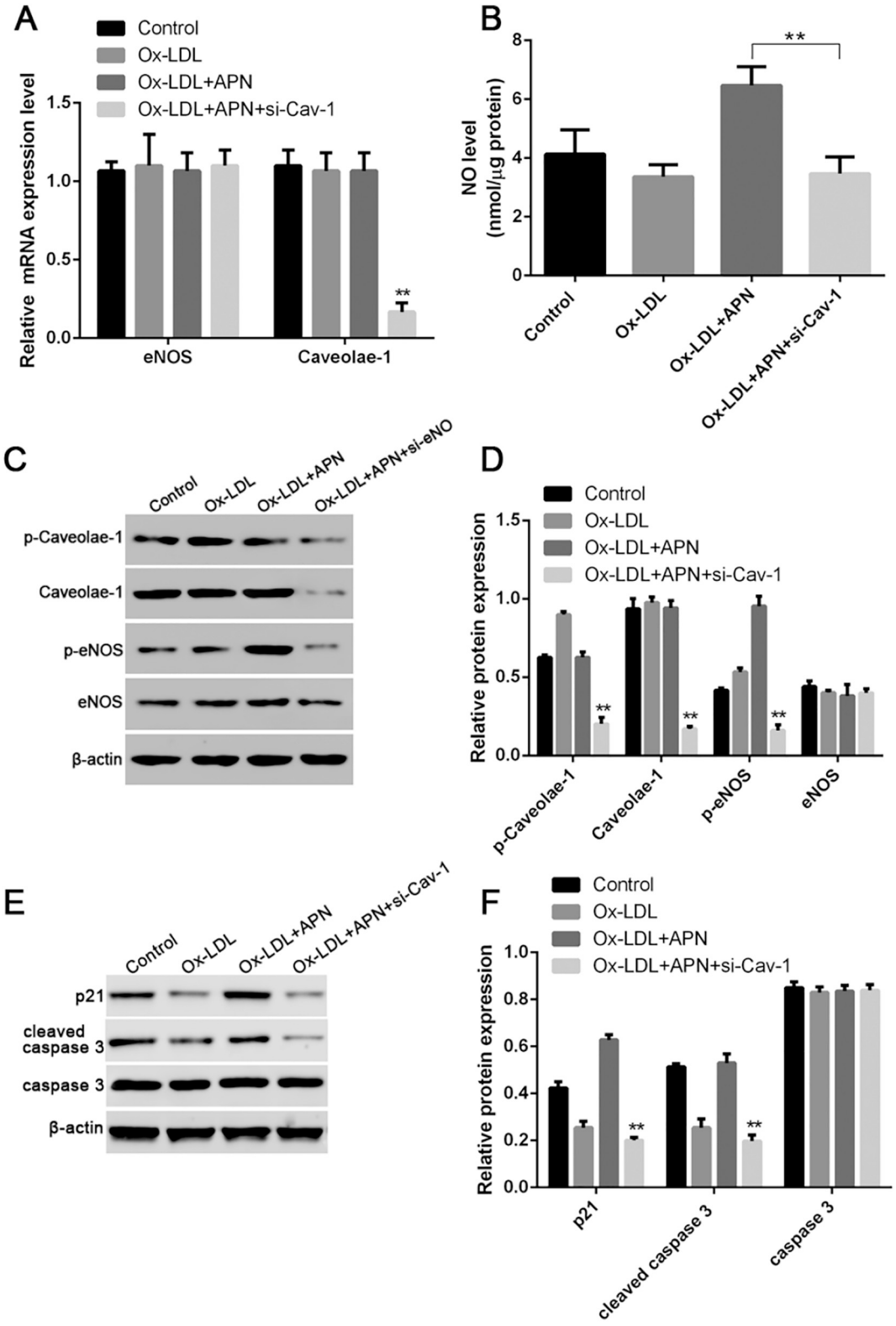

Fig. 7. Down regulation of Caveolae-1 expression could block NO secretion and upregulated expression of apoptosis related proteins induced by APN
A: RT-PCR results showed that the eNOS mRNA expression level was not affected after down regulation of Caveolae-1 expression; B: NO secretion induced by APN after down regulation of Caveolae-1 expression; C, D: Western blotting results showed that the expression levels of Caveolae-1 and p-Caveolae-1 were down-regulated after siRNA treatment, APN lost the function of up regulating p-eNOS expression, but had no significant effect on eNOS expression level; E, F: The protein levels of cleaved caspase 3 and p21 were also not up-regulated treated by APN after down regulation of Caveolae-1 expression.

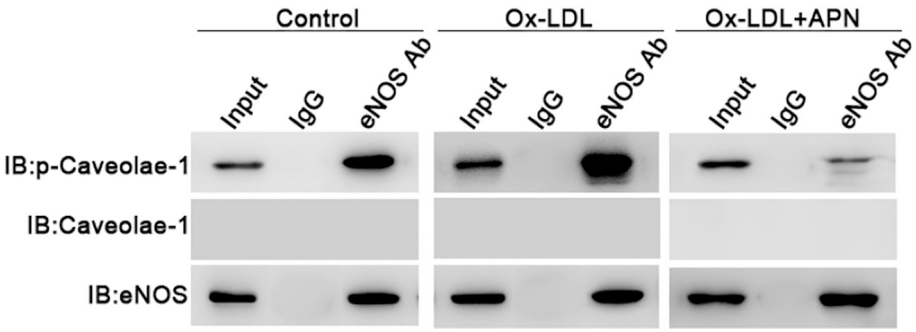

Fig. 8. eNOS combined with p-Caveolae-1 but not with Caveolae-1. ANP increases the uncombined eNOS level by reducing the p-Caveolae-1 level.

endothelial cells [24]. APN binds to its receptors and activates AMPK phosphorylation by APPL1. It phosphorylates eNOS through direct and indirect pathways and promotes NO secretion [25]. In this study, we found that both AdipoR1 and AdipoR2 were expressed in HAECs. In the HAECs with downregulated AdipoR1, the effect of APN on inhibiting the abnormal proliferation of HAECs induced by ox-LDL disappeared. However, in the HAECs with downregulated AdipoR2, APN still inhibited the abnormal proliferation of HAECs induced by ox-LDL. These indicated that APN inhibited the abnormal proliferation of HAECs induced by Ox-LDL through binding with AdipoR1 in HAECs.

Under physiological conditions, eNOS-derived NO is secreted by vascular endothelial cells and migrates to HAECs. NO regulates HAECs proliferation mainly through two pathways. Firstly, it induces the apoptosis of HAECs by promoting the production of cleavaged caspase 3. On the other hand, it increases the expression of p21 and inhibit the process of cell cycle [17,18]. We found that ox-LDL could promote cell proliferation by inhibiting p-eNOS level, down-regulating cleavaged caspase 3 and p21 expression in HAECs. APN did not affect the p-eNOS level in normal HAECs cells, but in ox-LDL treated HAECs, APN could promote the recovery of p-eNOS level, up-regulate the expression of cleavaged caspase 3 and p21, and inhibit the proliferation of HAECs. When siRNA was used to inhibit the expression of eNOS, the effect of APN on up-regulation of p-eNOS level was inhibited, and the function of up-regulation of cleavaged caspase 3 and p21 was also lost. APN could not inhibit the abnormal proliferation of HAECs cells induced by ox-LDL. These results indicated that APN inhibited ox-LDL induced HAECs proliferation by inducing NO secretion.

Previous studies have shown that the activity of eNOS is regulated by Caveolae-1. The phosphorylated Caveolae-1 can bind to eNOS to form a stable complex and inhibit the synthesis of NO. eNOS and Caveolae-1 dissociated and activated eNOS phosphorylation after dephosphorylation of Caveolae-1, which promoting NO production [25]. In this study, when siRNA was used to inhibit the expression of Caveolae-1, the function of APN in promoting eNOS phosphorylation and NO production was inhibited. APN could not inhibit HAECs proliferation induced by ox-LDL either. Co-immunoprecipitation results showed that eNOS had strong binding force with p-Caveolae-1, but almost had no combination with Caveolae-1 in HAECs. Inhibition of eNOS expression by siRNA did not affect the expression level of Caveolae-1 and its phosphorylation level. However, down regulation of Caveolae-1 with siRNA could not inhibit eNOS expression, but inhibit the eNOS phosphorylation. These results suggested that Caveolae-1 may be a key regulator of APN promoting eNOS phosphorylation in ox-LDL-induced HAECs. The regulation of phosphorylation and dephosphorylation of Caveolae-1 may directly affect the activation of eNOS and the production of NO, thereby regulating the abnormal proliferation of HAECs. Caveolae-1 may participate in the occurrence and development of cardiovascular diseases related to ox-LDL by regulating eNOS activity and NO production.

5. Conclusions

In a word, in this study, we found that APN could promote the up-regulation of cleavaged caspase 3 and p21 expression, and inhibit the abnormal proliferation of HAECs induced by ox-LDL through AdipoR1-mediated up regulation of eNOS phosphorylation and NO production. The regulation of phosphorylation and dephosphorylation of Caveolae-1 played a key regulatory role in this process. We will continue to analyze the molecular mechanism of Caveolae-1 in regulating APN signaling pathway in order to observe the possibility of APN in the treatment of cardiovascular diseases. Caveolae-1 may be one of the potential therapeutic targets.

Authors' contributions

Conceived and designed the experiments: Yongping Jia; Execution of experiments: Yan Lu, Xiaoying Gao, Ruihua Wang; Data analysis: Yan Lu, Jing Sun, Beibei Guo; Discussion of results: Yan Lu, Ruipeng Wei, Yongping Jia; Wrote and or critical reading of manuscript: Yan Lu and Yongping Jia.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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