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Depletion of CPEB1 protects against oxidized LDL-induced endothelial apoptosis and inflammation though SIRT1/LOX–1 signalling pathway

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ABSTRACT

Atherosclerosis (AS) is a chronic inflammatory disease that results from Oxidized low-density lipoprotein (Ox-LDL) induced endothelial dysfunction. Cytoplasmic polyadenylation element binding protein 1 (CPEB1) is closely related to the development of epithelial cells, but the role of CPEB1 in AS remains unknown. The RNA and protein levels of CPEB1 expression are increased by Ox-LDL exposure, which is abrogated by c-Jun amino-terminal kinase (JNK) inhibitor SP600125. CPEB1 small interfering RNA (siRNA) suppressed the oxidative stress, inflammation, and apoptosis. Furthermore, CPEB1 siRNA enhanced the sirtuin 1 (SIRT1) transcription levels in Ox-LDL-treated HUVECs. Co-Immunoprecipitation (Co-IP) assay showed that CPEB1 siRNA declined the ubiquitination of SIRT1, and SIRT1 siRNA enhanced the Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which were decreased by CPEB1 siRNA. In addition, LOX-1 and SIRT1 attenuated the protection of SIRT1 siRNA on Ox-LDL-induced oxidative stress. Therefore, our study revealed that CPEB1 depletion might play an anti-inflammatory and antiapoptotic role in Ox-LDL-induced apoptosis and inflammation though SIRT1/LOX-1 signalling pathway.

1. Introduction

Atherosclerosis (AS) is a chronic inflammatory disease that has dramatically increased in the world recently [1–3]. The pathogenesis of AS is quite complex with various theories and hypotheses, and it is well accepted that endothelial dysfunction is a driving force in the initiation and development of AS [4–6]. Therefore, it is critical to further elucidate the mechanism responsible for endothelial dysfunction in order to provide novel insights into therapies for AS.

The cytoplasmic polyadenylation element binding protein 1 (CPEB1) is a highly conserved RNA-binding protein, recruiting translation inhibitor to its purpose mRNA 3′ untranslated region and combining with the specific sequences to regulate the polyadenylation of mRNA [7–9]. CPEB1 is widely involved in biological processes, including cell cycle progression, cellular senescence and inflammation [9,10]. In addition, CPEB1 is also reported to function as a potential tumour suppressor in the angiogenesis of malignant tumour cells [11,12]. A recent study has indicated that CPEB1 was induced by various stimulators such as cytokines and lipopolysaccharide (LPS), and plays an important role in regulating inflammation. What's more, CPEB1 is a key regulator in human cytomegalovirus (HCMV) infection, which can cause serious diseases, including AS [13]. Thus, we speculated that CPEB1 might be involved in the development of AS.

Sirtuin 1 (SIRT1) is involved in regulating cellular physiological processes, such as growth and survival, stress resistance and inflammation [14]. The anti-atherosclerotic role of SIRT1 in the human endothelial cells is reported by protecting against endothelial from inflammation and promoting autophagy [15,16]. It is also reported that SIRT1 could negative regulate the Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which is a lectin-like receptor for Ox-LDL [17]. LOX-1 is generally located in human smooth muscle cells, macrophages and vascular endothelial cells [18]. In vascular endothelial cells, LOX-1 plays a vital role in initiating the formation and development of AS plaques by activating Nuclear factor-κB (NF-κB), affecting the generation of ROS, upregulating endothelium-derived nitric oxide (NO) and impairing endothelial dysfunction [19–23].

The present study aimed to examine the role of CPEB1 in an Ox-LDL-injured human umbilical vein endothelial cell (HUVEC) model. The protective effect of CPEB1 small interfering RNA (siRNA) on oxidative stress, inflammation and apoptosis were measured, and the potential related mechanisms were explored.

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2. Materials & methods

2.1. Cell culture

HUVECs were acquired from the American Type Culture Collection. HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, USA) supplemented with 10% foetal bovine serum (FBS, Thermo Fisher Scientific, USA), 100 U/ml penicillin (Millipore Sigma) and 100 μg/mL streptomycin (Invitrogen) at 37 °C in 5% CO_2 in a humidified chamber. To establish Ox-LDL-injured model, HUVECs were cultured for 24 h after treatment with Ox-LDL (0, 25, 50, 100 or 150 μg/mL, respectively). For investigating the involvement of ERK1/2, JNK and p38 MAPK signalling, HUVECs were treated with ERK1/2 inhibitor ulixertinib (BVD-523, 2 μM), JNK inhibitor SP600125(SP, 20 μM) and p38 MAPK inhibitor SB203580 (SB, 20 μM) before Ox-LDL stimulation.

2.2. Cell transfection

CPEB1 siRNA/scramble or SIRT1 siRNA/scramble were purchased from GenePharma Co. (Shanghai, China). The adenovirus overexpression vector containing the LOX-1 sequence and its negative control (vector) were synthesized by the pSilencer adeno 1.0-CMV System (Ambion) according to the manufacturer's protocol. Then, HUVECs were harvested for the subsequent experiments after transfection by Lipofectamine 3000 reagent (Invitrogen) for 48 h. The sequence of CPEB1 siRNA is: 5′-GCUCUGCAUGGAAUGCUAATT-3′, and the sequence of SIRT1 siRNA is: 5′-GUA UUU ACG UUC AAA UGA ATT-3'.

2.3. Measurement of reactive oxygen species (ROS) production

A dihydroethidium (DHE) probe was used to examine superoxide anion production. HUVECs were pre-treated with 5 μM of fluorescent probe DHE for 1 h in the dark at 37 °C before incubation with the Ox-LDL for 2 h. The fluorescence intensity was measured by using a SpectraMax M5 fluorimeter at 540 nm excitation and 590 nm emission. Fluorescence intensity was determined with excitation and emission wavelengths of 493 nm and 522 nm, respectively.

2.4. Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojido, Japan) was used to detect cell proliferation according to the manufacturer's protocol. Briefly, 1×10^4 cells were seeded into 96-well plates and incubated for 24 h, and then, 10 μl CCK-8 solution was supplemented for each well. The cells were incubated at 37 °C for 2 h. The absorbance at 450 nm was measured using a SpectraMax M5 fluorimeter every day.

2.5. Cell apoptosis assay

The apoptotic rate was quantitatively analysed by the Annexin V-FITC/PI Apoptosis Detection Kit (BioVision, Milpitas, CA, USA). After Ox-LDL stimulation for 5 h, HUVECs (5×10^5 cells/well) were washed twice by cold phosphate buffered saline (PBS) and re-suspended in 200 μl buffer. Then, 10 μl Annexin V-FITC and PI were added and incubated at room temperature in the dark for 10 min. The apoptotic cells (Annexin-V positive and PI-negative) were measured by a flow cytometer (BD Bioscience, USA).

2.6. Quantitative real-time PCR (qPCR)

Total RNA of HUVECs was isolated by using the miRNeasy Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. cDNA was synthesized by using a random primer (Sangon Biotech, Shanghai, China). SYBR™ Select Master Mix (Thermo Fisher Scientific) and Applied Biosystems 7500 Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA) were used to detect the relative expression levels of CPEB1, iNOS, SIRT1, TNF-α, IL-6 and IL-8. GAPDH was used as an internal control. The relative expression of each gene was calculated and normalized by using the $2^{-\Delta\Delta CT}$ method.

2.7. Enzyme-linked immunosorbent assay (ELISA)

HUVECs were induced by 5 μg/mL Ox-LDL for 12 h. The levels of NO, TNF-α, IL-6 and IL-8 were detected by ELISA kit (R&D Systems, Abingdon, UK) according to the protocols of the manufacturer. All absorbances of the samples were determined by a microplate reader (Multiskan Spectrum, Thermo, MA, USA). Cytokine levels were standardized as protein concentrations.

2.8. NO assay

Griess reagent (R&D Systems, Abingdon, UK) and the Nitric Oxide Colorimetric Assay Kit (Biovision, San Francisco, USA) were used to detect the NO levels of HUVECs, and absorbance was recorded by Thermo Scientific Varioskan Flash (Thermo LabSystems, Inc., Philadelphia, PA) at 540 nm according to the manufacturer's protocol. NO production is expressed as mol/L.

2.9. Western blotting assay and caspase-3 activity assay

HUVECs was pre-treated with ulixertinib (BVD-523, 2 μM), SP600125(SP, 20 μM) and SB203580 (SB, 20 μM) before Ox-LDL stimulation. Total protein was extracted using a RIPA lysis buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (P0013B; Beyotime, Jiangsu, China), and the concentration was detected using bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). Protein was separated by using SDS-PAGE (Beyotime Biotechnology, Shanghai) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was cultured overnight with primary antibody at 4 °C overnight after being blockaded in 5% skim milk. The horseradish peroxidase (HRP) conjugated secondary antibody (Abcam), which combines with the membrane, was further cultured for 1.5 h at 37 °C. Protein imprinting was observed by enhanced chemiluminescence detection kit (Biorbyt, Shanghai, China) and the relative expression of protein was quantified by image lab software (Bio-Rad, Hercules, CA, USA).

Primary antibodies (Abcam) against CPEB1 (ab3465, 1:1000), cleaved caspase-3 (ab32042, 1:1000), SIRT1 (SIRT1, 1:1000), LOX-1 (ab60178, 1:1000) and GAPDH (ab8245, 1:2000) at 4 °C overnight. After washing, secondary antibodies labelled with HRP were incubated at room temperature for 1 h. Goat antirabbit IgG (ab6721, 1:5000) and goat anti-mouse IgG (ab6789, 1:5000) were used as secondary antibodies (Abcam).

2.10. Co-Immunoprecipitation (Co-IP) assay

A Co-IP assay was performed using lysates from HUVECs that were transfected with CPEB1 siRNA/scramble or SIRT1 siRNA/scramble. In short, the transfected HUVECs were isolated in 500 mL Co-IP buffer and incubated with 5 μg primary antibody at 4 °C for 2 h. A total of 40 μl 50% protein A- Sepharose syrup was added to each sample and incubated at 4 °C for 4 h. The particles were washed with PBS and resuspended in 0.5 mL Tri Reagent (Sigma Aldrich). The coprecipitation process of elution in aqueous solution was analysed by Western blotting assay.

2.11. Statistical analysis

All experiments composed three different experiments with at least

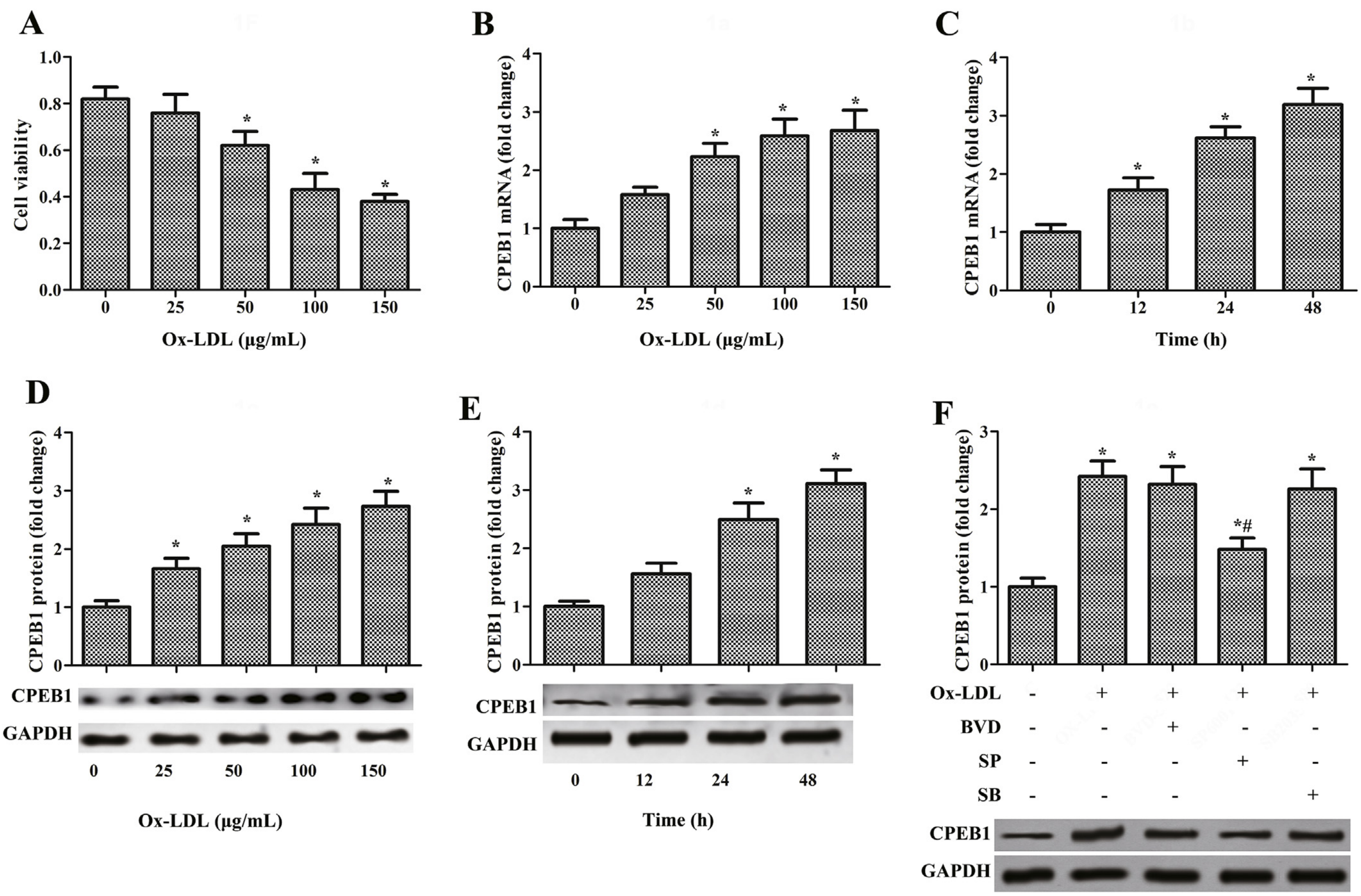

Fig. 1. Ox-LDL-induced increased CPEB1 expression through JNK signalling pathway. Human umbilical vein endothelial cells (HUVECs) were cultured and treated with (0, 25, 50, 100 or 150 μg/mL) oxidized LDL (Ox-LDL) for 24 h, or with 100 μg/mL Ox-LDL for 0,12, 24 or 48 h. (A) The cell viability of HUVECs was assessed by CCK-8 assay. (B) The CPEB1 mRNA levels of HUVECs were detected by quantitative reverse-transcriptase PCR. (C) The CPEB1 mRNA levels of HUVECs were detected by quantitative reverse-transcriptase PCR. (D) The protein expression level of CPEB1 were detected by Western blotting. (E) The protein levels of CPEB1 in HUVEC groups were detected by Western blotting. (F) HUVECs were treated with 100 μg/mL Ox-LDL for 24 h and BVD, SP or SB, respectively. The protein levels of CPEB1 in HUVEC groups were detected by Western blotting. BVD: ERK1/2 inhibitor BVD-523; SP: JNK inhibitor SP600125; SB: p38 MAPK inhibitor SB203580. β-actin as control.

$^{*}p < 0.05$ vs con (without Ox-LDL treatment), $^{\#}p < 0.05$ vs Ox-LDL group.

three replicates. Statistical analysis was carried out using SPSS 22.0 software (SPSS Inc., USA). Comparisons of data among groups were performed by one-way or two-way ANOVA followed by Tukey's post-hoc test as appropriate. The data are all indicated as the mean ± SEM. The result value of $^{*}p < 0.05$ or $^{\#}p < 0.01$ was considered statistically significant.

3. Results

3.1. *Ox-LDL increased CPEB1 expression through JNK signalling pathway*

As shown in Fig. 1A, 50, 100 and 150 μg/mL Ox-LDL induces a dose-dependent reduction of the cell viability in HUVECs. The mRNA and protein expression levels of CPEB1 were significantly increased by Ox-LDL in a dose- and time-dependent manner ($p < 0.05$) (Fig. 1B–E). As shown in Fig. 1F, three signalling inhibitors, including ulixertinib (BVD-523), JNK inhibitor SP600125(SP) and p38 MAPK inhibitor SB203580 (SB), were used to detect the induced pathway of Ox-LDL in HUVECs. As shown, the CPEB1 protein expression level was significantly decreased by SP, which suggests that Ox-LDL increased CPEB1 expression through the JNK signalling pathway.

3.2. *Depletion of CPEB1 suppressed Ox-LDL-induced oxidative stress and inflammation*

In order to investigate the effects of CPEB1 on Ox-LDL-induced oxidative stress and inflammation in HUVECs, CPEB1 siRNA was transfected into HUVECs. As shown in Fig. 2A, the CPEB1 protein expression level was reduced by CPEB1 siRNA ($p < 0.05$). As indicated in Fig. 2B–D, CPEB1 siRNA significantly decreased the Ox-LDL-induced ROS generation levels, iNOS mRNA level and NO production ($p < 0.05$). In addition, CPEB1 siRNA significantly decreased the mRNA levels and concentrations of TNF-α, IL-6 and IL-8 ($p < 0.05$) (Fig. 2E and F).

3.3. *Depletion of CPEB1 suppressed Ox-LDL-induced apoptosis*

Next, we explored the effect of CPEB1 siRNA on the apoptosis of Ox-LDL-treated HUVECs. As indicated in Fig. 3A and B, CPEB1 siRNA alleviates the inhibitory effect of Ox-LDL on cell viability ($p < 0.05$) (Fig. 3A) and the Ox-LDL-induced apoptosis rate ($p < 0.05$) (Fig. 3B). Moreover, the suppressed cleaved caspase-3 levels, which were induced by Ox-LDL treatment, were alleviated by CPEB1 siRNA ($p < 0.05$) (Fig. 3C).

Silence of CPEB1 downregulated the expression of LOX-1 through deubiquitinating SIRT1 in HUVECs.

The previous study reported that CPEB1 directly regulated SIRT1 to

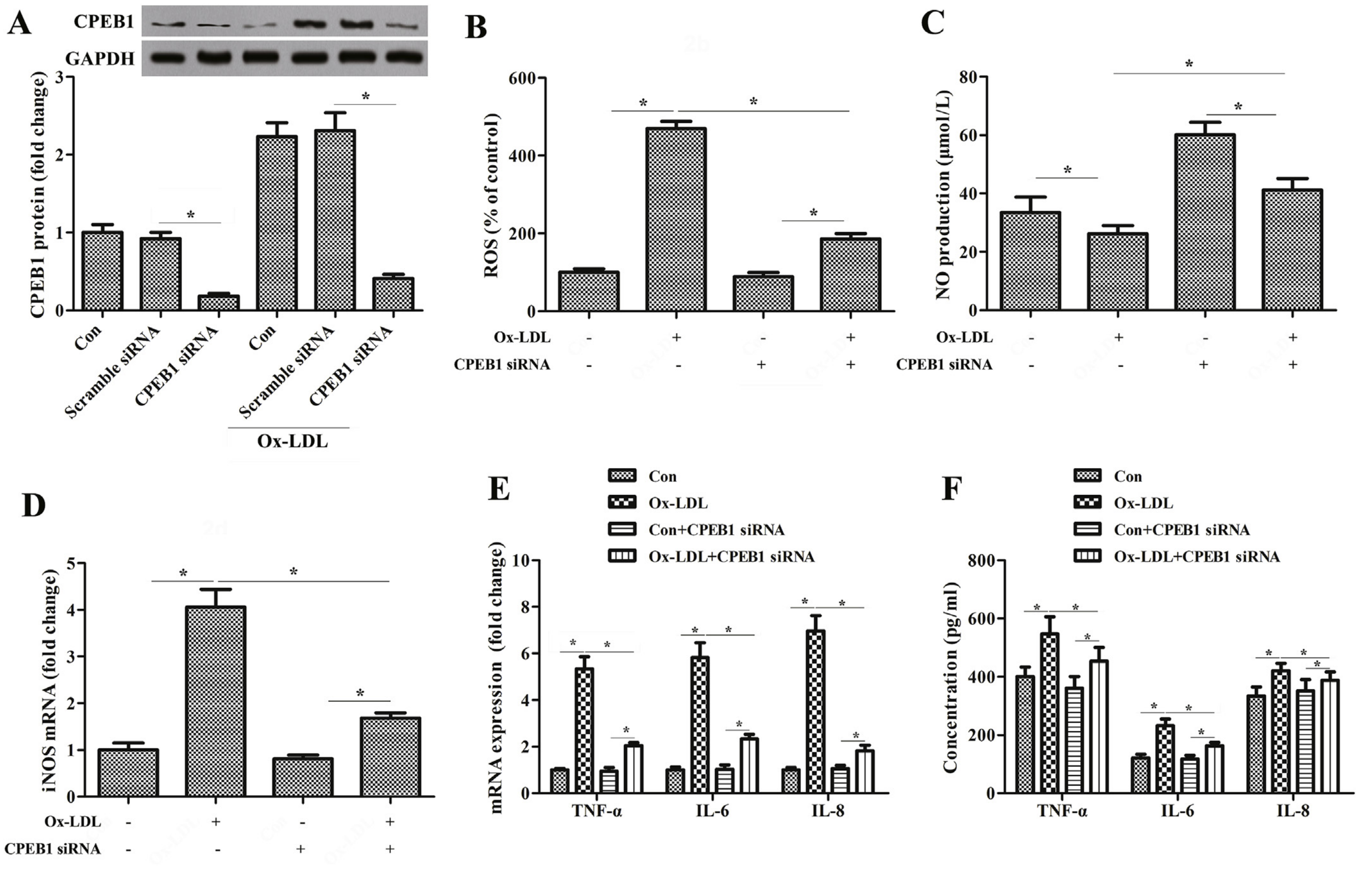

Fig. 2. Depletion of CPEB1 suppressed Ox-LDL-induced oxidative stress and inflammation. HUVECs were transfected with CPEB1 siRNA/scramble and 100 μg/mL Ox-LDL. HUVECs without Ox-LDL treatment as control. CPEB1 scramble was used as negative control. (A) The protein levels of CPEB1 in HUVEC groups were detected by Western blotting. (B) Fluorescence distribution of DHE oxidation expressed as a percentage of increased intensity. (C) The NO production (mol/L) of HUVECs was detected by NO assay. (D) The iNOS mRNA levels of HUVECs were detected by quantitative reverse-transcriptase PCR. (E) The mRNA levels of TNF-α, IL-6 and IL-8 in HUVECs were detected by quantitative reverse-transcriptase PCR. (F) The concentrations of IL-6, IL-1β and TNF-α in HUVECs were detected by enzyme-linked immunosorbent assay. *$p < 0.05$.

mediate cancer stemness in hepatocellular carcinoma (HCC) [10]. Thus, we studied SIRT1 in our study. The results showed that CPEB1 siRNA had no function on the transcription of SIRT1 mRNA levels, but dramatically improved SIRT1 protein levels in Ox-LDL-treated HUVECs ($p < 0.05$) (Fig. 4A and B). Immunoprecipitation assay indicated that CPEB1 siRNA declined the ubiquitination of SIRT1 in Ox-LDL-treated HUVECs (Fig. 4C). In addition, the protein expression level of LOX-1 was significant decreased by CPEB1 siRNA, and this inhibitory effect of

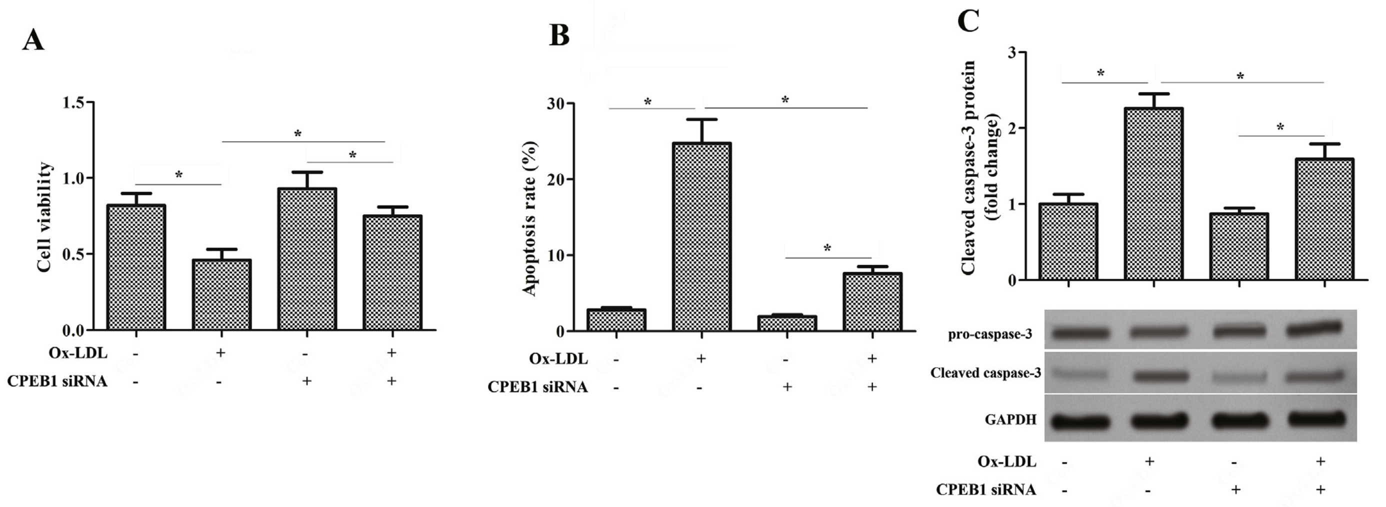

Fig. 3. Depletion of CPEB1 suppressed Ox-LDL-induced apoptosis. HUVECs pretreat by Ox-LDL were transfected with CPEB1 siRNA/scramble. (A) The cell viability of HUVECs was assessed by CCK-8 assay. (B) The apoptosis rate of HUVECs was detected by Annexin V-FITC/PI assay. (C) The protein levels of pro-caspases-3 and Cleaved caspases-3 in HUVECs were detected by Western blotting. *$p < 0.05$.

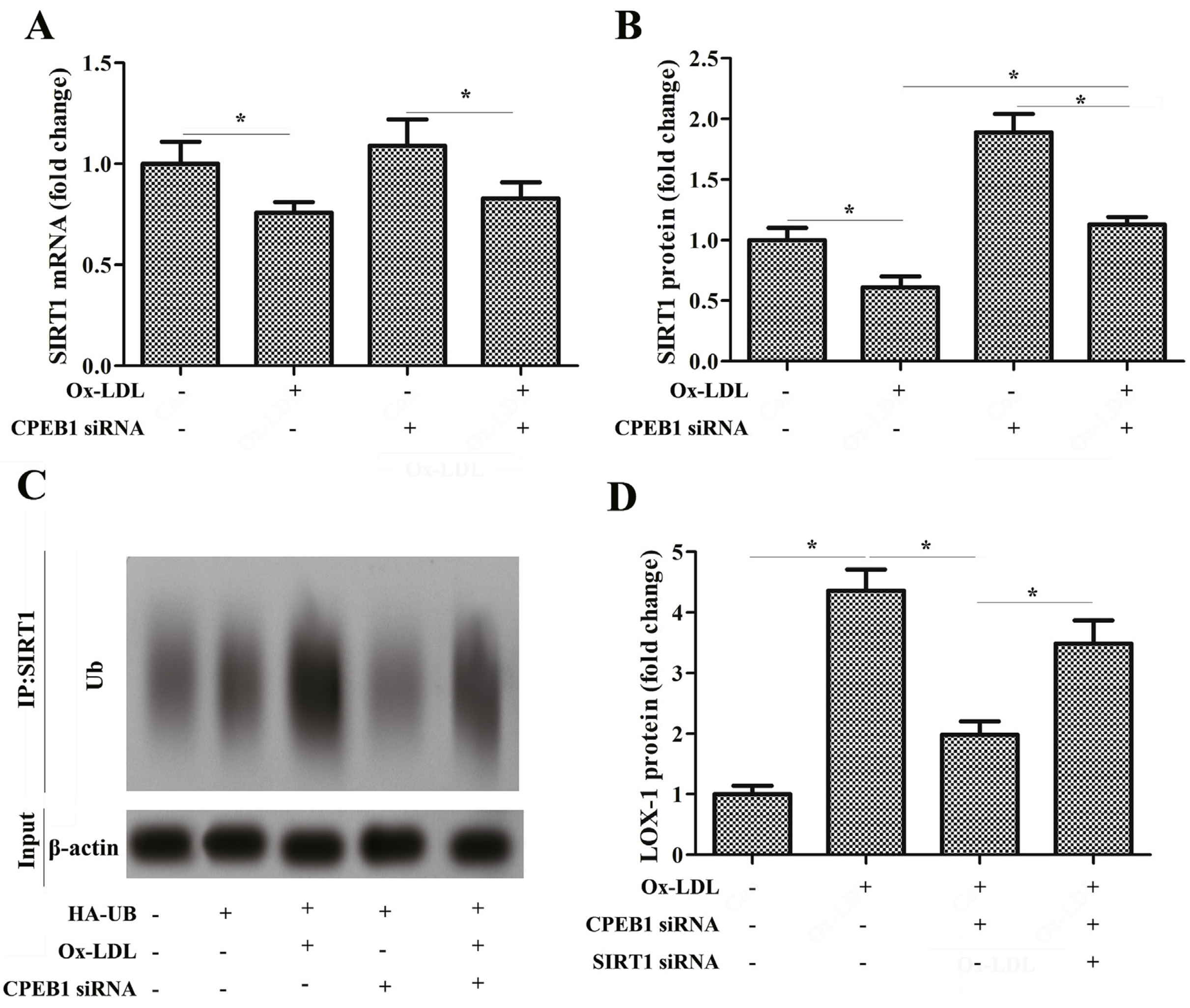

Fig. 4. Silence of CPEB1 downregulated the expression of LOX-1 through deubiquitinating SIRT1 in HUVECs. HUVECs pretreat by Ox-LDL were transfected with CPEB1 siRNA/scramble. (A) The mRNA levels of SIRT1 in HUVECs were detected by qPCR. (B) The protein levels of SIRT1 in HUVECs were detected by Western blotting. (C) HUVECs were transfected with hemagglutinin (HA)-ubiquitin for 24 h before treatment with Ox-LDL. The total cell lysates were immunoprecipitated by an SIRT1 antibody and studied by western blotting. (D) The protein levels of LOX-1 in HUVECs were detected by Western blotting. *$p < 0.05$.

CPEB1 siRNA on LOX-1 was attenuated by SIRT1 siRNA.

3.4. LOX-1 and SIRT1 participate in CPEB1 silence involved cell protection

Next, we confirmed the involvement of LOX-1 and SIRT1 in CPEB1 regulated Ox-LDL-induced injury. As shown in Fig. 5A, SIRT1 siRNA transfection or LOX-1 siRNA transfection significantly decreases the elevated cell viability induced by CPEB1 siRNA ($p < 0.05$). SIRT1 siRNA transfection or LOX-1 siRNA transfection significantly increased mRNA levels of the apoptosis rate and inflammatory cytokines in Ox-LDL-treated HUVECs transfected with CPEB1 siRNA ($p < 0.05$) Fig. 5B–E).

4. Discussion

Atherosclerotic plaque rupture is the main cause of most acute vascular events, such as stroke and acute myocardial infarction [2,24,25]. Ox-LDL plays an extremely important role in vascular endothelial dysfunction and structural damage, especially in the occurrence and development of AS in cardiovascular diseases [26,27]. In our study, 100 μg/mL Ox-LDL was used to-treat HUVECs, and our data showed the expression of CPEB1 was significantly increased by Ox-LDL treatment through the JNK signalling pathway, which is consistent with the results of Chan et al., who reported that the expression and phosphorylation of CPEB1 was increased by LPS stimulation in a JNK-dependent manner in rat primary astrocytes [28]. Besides, elevated CPEB1 expression was also found by EGF and pro-inflammatory cytokines (i.e., IL-1β and TNF-α) [29–31].

Inflammation and endothelial disease can promote plaque rupture, so the prevention of inflammation and inhibition of endothelial dysfunction are the key to slowing down AS [32,33]. CPEB1 is known to play a role in a variety of molecular pathways, such as cell proliferation, differentiation and apoptosis [34–36]. Notably, CPEB1 siRNA was also indicated to reduce oxidative stress and inflammatory responses in LPS-treated astrocytes [28]. Ivshina et al. reported that CPEB1 depletion decreased the production of pro-inflammatory cytokines in mouse embryo fibroblasts [37]. In accordance with these studies, the present study showed that the depletion of CPEB1 suppressed Ox-LDL-induced oxidative stress, inflammation and apoptosis in HUVECs. While Li et al. [30] implied that CPEB1 was induced by pro-inflammatory cytokines. Thus, CPEB1 may regulate inflammation through a positive feedback loop.

SIRT1, a member of the NAD$^+$-dependent deacetylases, plays an important role in regulating cellular physiological processes. SIRT1 enhances autophagic flux in cardiomyocytes and is recognized as a novel target to prevent human endothelial pathology [38–40]. Besides,

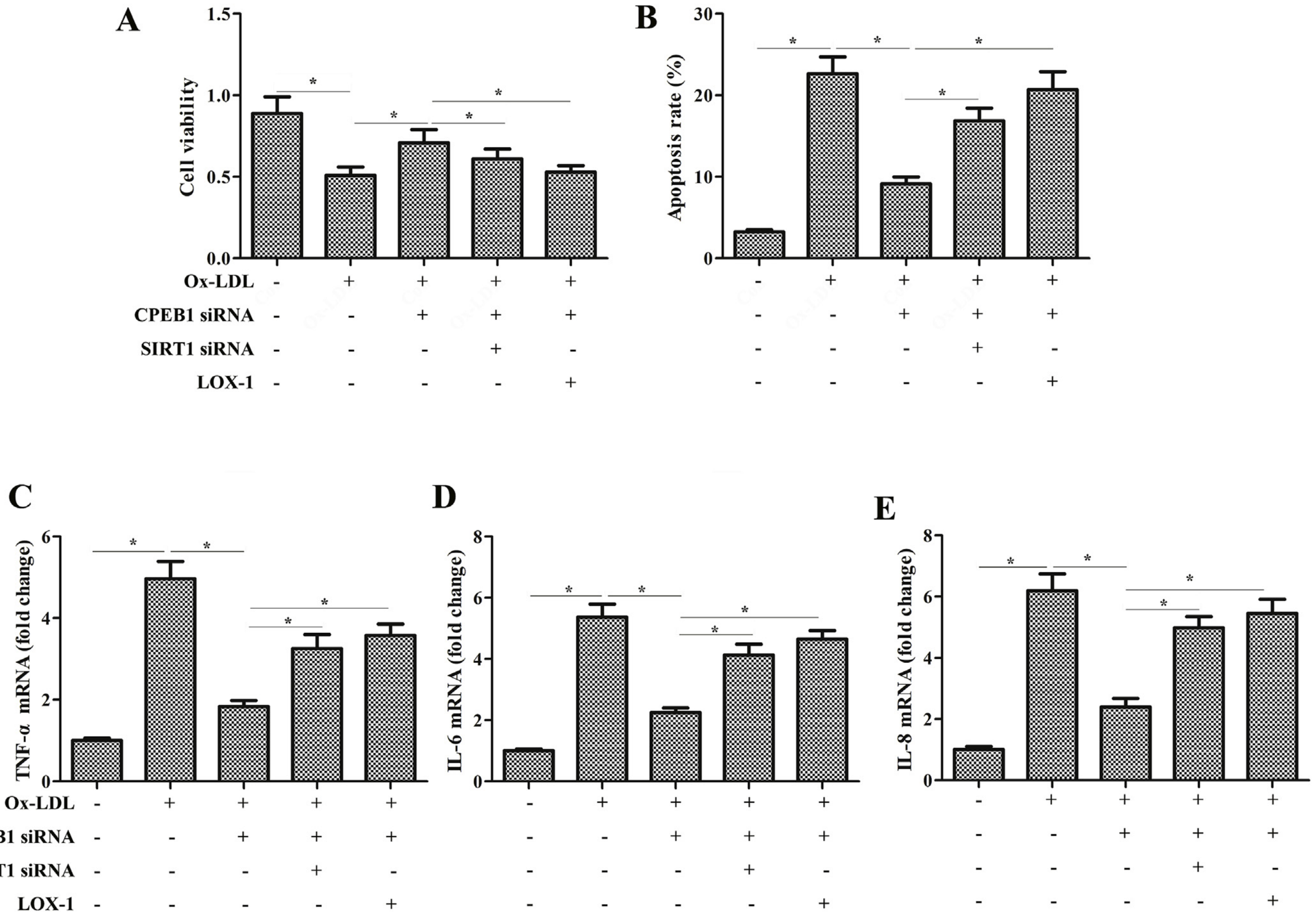

Fig. 5. LOX-1 and SIRT1 participate in CPEB1 silence involved cell protection. HUVECs pretreat with Ox-LDL were transfected with CPEB1 siRNA/scramble or SIRT1 siRNA/scramble, or LOX-1 adenovirus overexpression vector/negative vector. (A) The cell viability of HUVECs was assessed by CCK-8 assay. (B) The apoptosis rate of HUVECs was detected by Annexin V-FITC/PI assay. (C–E) The mRNA levels of TNF-α, IL-6 and IL-8 in HUVECs were detected by quantitative reverse-transcriptase PCR. $^{*}p < 0.05$.

SIRT1 is also reported to inhibit the degeneration of human endothelial cells through the regulation of anti-apoptotic genes, thereby reducing endothelial inflammation and promoting survival [41]. CPEB1 was proved to directly target the 3′-UTR of SIRT1, control poly(A) tail length and suppress its translation, and CPEB1 depletion resulted in dramatically increased SIRT1 expression in cancer stem cells (CSCs) [4,10,42]. A recent report presented that SIRT1 reduces LOX-1 expression and diminishes uptake of Ox-LDL in atherogenesis [43,44]. Our data showed that CPEB1 silence downregulated the expression of LOX-1 though deubiquitinating SIRT1 in Ox-LDL-treated HUVECs.

In summary, our present study demonstrated that CPEB1 levels were significantly increased by Ox-LDL treatment, and CPEB1 knockdown downregulated the expression of LOX-1 to inhibit the oxidative stress, inflammation and apoptosis though deubiquitinating SIRT1 in Ox-LDL-treated HUVECs. This study found novel insights into the anti-inflammatory and anti-apoptotic roles of CPEB1 in development of AS, which suggests that CPEB1 might be a promising target for the treatment of AS.

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