NEDD4 attenuates oxidized low-density lipoprotein-induced inflammation and dysfunction in vascular endothelial cells via regulating APEX1 expression

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Abstract. Atherosclerosis chiefly results from inflammation as well as vascular endothelial cell dysfunction. Methylation levels of neuronally expressed developmentally downregulated 4 (NEDD4) were found to be fortified in atherosclerosis patients and NEDD4 deficiency enhanced vascular calcification. However, the exact function of NEDD4 in inflammation and vascular endothelial dysfunction remains to be elucidated. In the present study, CCK-8 assay was used to estimate cell viability. Reverse transcription-quantitative PCR was adopted to examine the expression of NEDD4, inflammation-associated enzymes and apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1). Western blotting was used to test NEDD4, endothelial nitric oxide synthase, inducible nitric oxide synthase and APEX1 protein levels. Cytotoxicity was detected by a lactate dehydrogenase (LDH) kit. Reactive oxygen species level was tested by a corresponding kit. Vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 contents were examined with ELISA. Cell adhesion assays evaluated the adhesion of endothelial cells. Co-immunoprecipitation assay was used to test the relationship between NEDD4 and APEX1. The data revealed that NEDD4 expression rapidly declined in oxidized low density lipoprotein (ox-LDL)-induced human umbilical vein endothelial cells (HUVECs). Following NEDD4 overexpression, the active damage, inflammatory release and endothelial cell dysfunction in ox-LDL-induced HUVECs were attenuated. After co-transfection of APEX1 interference plasmids and NEDD4 overexpression plasmids, cell damage, inflammatory release and endothelial cell dysfunction in ox-LDL-induced HUVECs were improved again. Taken together, NEDD4 attenuated ox-LDL-induced inflammation and endothelial dysfunction by regulating APEX1 expression.

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Introduction

Atherosclerosis (AS) is a chronic inflammatory disease detrimental to human health and a common cause of mortality among the elderly. Inflammation occupies a pivotal position during the process of AS (1). The symptoms of AS are mainly manifested as inadequate blood supply, dizziness, memory loss and even myocardial infarction and sudden death in severe cases of coronary AS. Previous therapies focus on the prevention of risk factors, such as high cholesterol and high blood pressure (2). However, the incidence rate of AS complications remains high. Numerous reports about epidemiological and clinical studies have shown that abnormally high concentrations of oxidized low density lipoprotein (ox-LDL) play a central role in the development of AS (3-5).

As a risk factor of AS, ox-LDL has been found to damage vascular endothelial cells, bring about endothelial dysfunction and contribute to the production of inflammatory cytokines, all of which are engaged in and drive the process of AS (6). Ox-LDL downregulates insulin-like growth factor-1 receptor in human smooth muscle cells, but overexpression of dominant-negative NEDD4 prevents ox-LDL-induced downregulation of insulin-like growth factor-1 receptor, which suggests that neuronally expressed developmentally downregulated 4 (NEDD4) is a ubiquitin ligase that mediated receptor downregulation (7).

NEDD4, serves as an essential member of HECT domain E3 ligase family in eukaryotes (8) and has been proved to play a prominent part in various cellular processes through the ubiquitination-mediated degradation of multiple substrates (9). Various cardiovascular diseases are strongly dependent on the ubiquitin proteasome system (10). Previous studies found that aberrant activity of the TGF-β signaling pathway may contribute to the process of the calcification of vascular smooth muscle cells (VSMCs) (11,12). In addition, the phosphorylated Smad1 in BMP/TGF-β signaling could be regulated by NEDD4 E3 ligase which serves as a key inhibitor (13). As a result, the role of NEDD4 in the progression of vascular calcification has been confirmed. Furthermore, the methylation levels of the NEDD4 gene promoter are significantly increased in patients

with AS and NEDD4 deficiency enhance vascular calcification (14). However, there is no direct evidence for a specific link between NEDD4 and AS. On the basis of BioGRID database (https://thebiogrid.org), NEDD4 could interact with APEX1.

Apurinic-apyrimidinic endonuclease-1 (APEX1) is a multifunctional protein involved in the DNA damage response and is expressed in a variety of human tissues (15). The abnormal expression of APEX1 can mediate several physiological processes (16). Meanwhile, APEX1 has been reported to attenuate oxidative stress in ox-LDL-induced endothelial cells (17). Recent study has shown that APEX1 is able to inhibit AS-induced foam cell formation (18). However, whether APEX1 can bind to NEDD4 and participate in ox-LDL-induced endothelial cell inflammation and endothelial dysfunction remains to be elucidated.

Hence, the present study sought to investigate whether NEDD4 can act on ox-LDL-induced inflammation and endothelial dysfunction in vascular endothelial cells by regulating APEX1.

Materials and methods

Bioinformatics tools. The relationship between NEDD4 and APEX1 was predicted by BioGRID database (https://thebiogrid.org).

Cell culture and treatment. DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS in an incubator at 37˚C with 5% CO_2 was used to culture human umbilical vein endothelial cells (HUVECs) from Fudan IBS Cell Center. HUVECs between the third and fifth passages were selected for experiments. Increasing doses of ox-LDL (25, 50, 100 and 200 μg/ml) were to treat cells for 24 h at 37˚C.

RPMI 1640 medium (Thermo Fisher Scientific, Inc.) with 20% FBS at 37˚C in a 5% CO_2 incubator with 90% humidity was to culture U937 monocytes (CRL.1593.2) procured from American Type Culture Collection.

Cell transfection. The overexpression plasmid of NEDD4 (4 μg) and negative control plasmid (empty vector; 4 μg) were purchased from Addgene, Inc. and transfected into HUVECs. The short interfering (si)RNA-APEX1-1, siRNA-APEX1-1 and negative control plasmid were obtained from Nanjing Cobioer Gene Technology Co., Ltd. Target sequence of siRNA-APEX1-1 was AGGGTACAAGGCACTATGAAATG, target sequence of siRNA-APEX1-2 was GGCACTATGAAATGATCTAGTTT and target sequence of siRNA-NC was UAGCGACUAAACACAUCAA. HUVECs were seeded into 6-well plates (2x10^5 cells/ml). HUVECs were transfected with 20 μM siRNA-APEX1-1 and siRNA APEX1-2 and negative control siRNA using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C following the directions of the manufacturer. Subsequent experimentation was performed at 24 h after cell transfection.

Cell counting Kit-8 assay. CCK-8 solution (10 μl; GlpBio) was added to HUVECs grown in 96-well plates at 37˚C for 2 h. OD450 nm value was examined with a microplate reader (BioTek Instruments, Inc.).

Reverse transcription-quantitative (RT-q) PCR. HUVECs suspension was placed in a 6-well plate (2x10^5 cells/ml). By means of reverse transcription kit (Roche Diagnostics), cDNA was generated from the extracted RNA from HUVECs dependent on TRIzol® reagent (Thermo Fisher Scientific, Inc.) in accordance with the guidance from the manufacturer. The expression levels of target mRNAs in the HUVECs were subsequently analyzed through RT-qPCR using SYBR-Green I dye (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocols. The thermocycling conditions were: Initial denaturation at 95˚C for 5 min; 38 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 30 sec. The data were analyzed using $2^{-\Delta\Delta Cq}$ method (19) and normalized to GAPDH gene expression. The reactions were performed in duplicate for each sample. The primer sequences used were as follows: TNF-α forward, 5'-CTGGGCAGGTCTACTTTGGG-3' and reverse, 5'-CTGGAGGCCCCAGTTTGAAT-3'; IL-1β forward, 5'-AGCCATGGCAGAAGTACCTG-3' and reverse, 5'-TGAAGCCCTTGCTGTAGTGG-3'; IL-6 forward, 5'-GTCCAGTTGCCTTCTCCCTGG-3' and reverse, 5'-CCCATGCTACATTTGCCGAAG-3'; NEDD4 forward, 5'-GAGCTCAGCTTAAAGGTCGC-3' and reverse, 5'-TCTCTGTCCGTAGACAGGCT-3'; APEX1 forward, 5'-GCAGATACGGGGTTGCTCTT-3' and reverse, 5'-ATTTTTACCGCGTTGCTCGC-3'; APEX1 forward, 5'-GCAGATACGGGGTTGCTCTT-3' and reverse, 5'-ATTTTTACCGCGTTGCTCGC-3'; GAPDH forward, 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse, 5'-GCGCCCAATACGACCAAATC-3'.

Western blotting assay. After washing twice with cold PBS, total protein was extracted from HUVECs using RIPA lysis buffer. The protein concentration was measured with a bicinchoninic acid protein (BCA) assay kit (Beyotime Institute of Biotechnology). PVDF membranes (MilliporeSigma) were used to move the extracted proteins (50 μg/lane) separated by 10% SDS-PAGE. Skimmed milk (5%) was to impede the membranes for 2 h at room temperature following washing with TBST containing 0.1% Tween-20. Treatment with primary antibodies for one night and secondary antibody for 1.5 h was performed at 4˚C and at room temperature respectively. Finally, protein bands were visualized using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) and ImageJ software (version 1.43; National Institutes of Health) was used for protein band analysis. NEDD4 (cat. no. 2740; 1:1,000; Cell Signaling Technology), eNOS (cat. no. ab300071; 1:1,000; Abcam), iNOS (cat. no. ab178945; 1:1,000; Abcam), APEX1 (cat. no. ab189474; 1:1,000; Abcam) and GAPDH (cat. no. ab9485; 1:2,500; Abcam) antibodies were used in this study.

Lactate dehydrogenase (LDH) assay. HUVECs were treated with different concentrations of ox-LDL (25, 50, 100 and 200 μg/ml) for 24 h at 37˚C and then transferred into 96-well flat bottom plate. An LDH assay kit (Shanghai Coibo Biotechnology Co., Ltd.) was used to test the released LDH in the media based on the instructions of the manufacturer. The maximum amount of LDH release was determined and the absorbance was measured at 490 nm with the aid of a Synergy HT Microplate Reader (BioTek Instruments, Inc.).

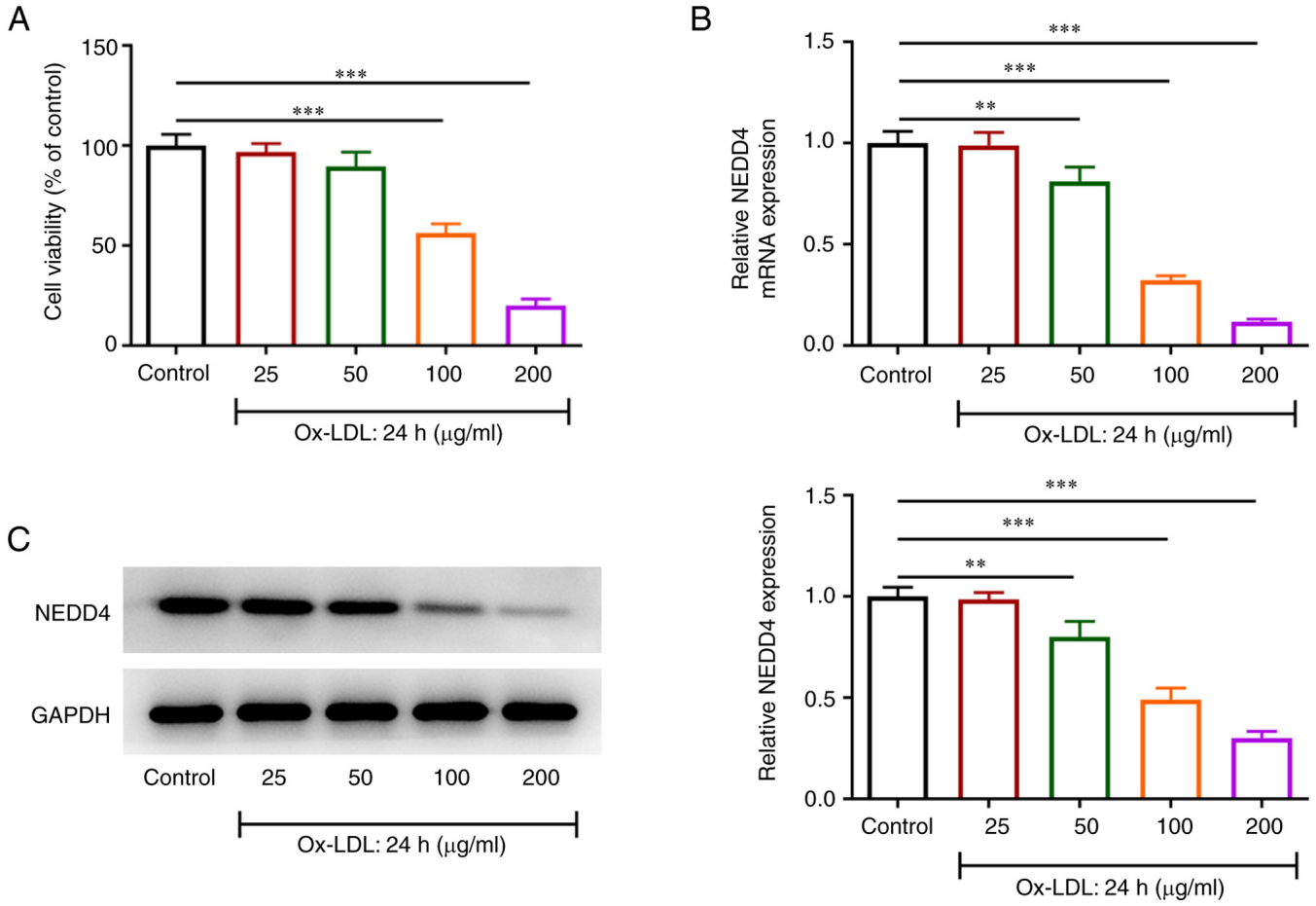

Figure 1. NEDD4 expression declines in ox-LDL-induced endothelial cells. (A) Cell activity of HUVECs treated by ox-LDL was assessed by CCK-8. The expression of NEDD4 in ox-LDL-induced endothelial cells was detected by (B) Reverse transcription-quantitative PCR and (C) western blotting assay. **P<0.01 and ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; ox-LDL, oxidized low density lipoprotein; HUVECs, human umbilical vein endothelial cells.

Measurement of reactive oxygen species (ROS). ROS generation was detected using a ROS ELISA kit (F06100; Shanghai Yanjin Biotechnology Co., Ltd.). Briefly, after the cells were treated, the supernatant was taken and the level of ROS was detected according to the ROS kit instructions. A microplate reader was used to measure the OD value of each well at the wavelength of 450 nm and the ROS concentration calculated according to the standard curve. The ratio of ROS concentration in each group to that in the control group was the relative ROS level.

ELISA. The antigens were solubilized by 50 mM of carbonate buffer (pH 9.6). The solution at a concentration of 10-20 μg/ml was added to a 96-well enzyme labeling plate overnight at 4˚C. Finally, the levels of vascular cell adhesion molecule (VCAM)-1 (cat. no. E-EL-H5587c) and intercellular adhesion molecule (ICAM)-1 (cat. no. E-EL-H6114) were determined by an ELISA kit (Elabscience Biotechnology, Inc.).

Monocyte adhesion assay. TNF-α (10 ng/ml) was used to pretreat HUVECs for 12 h at 37˚C. U937 monocytes were labelled with calcein-acetoxymethyl ester (Abcam) and cultured for 20 min at 37˚C. Then, the labelled U937 cells ($5x10^5$) were added to the culture media containing $1x10^5$ HUVECS for 2 h at 37˚C followed by washing 3 times with PBS. The estimation of attached green cells was conducted using an Olympus fluorescence microscope (magnification, x100; Olympus Corporation). Cells from five random high power fields for each well were counted to assess the average number of adherent cells.

Co-immunoprecipitation (Co-IP) assay. Antibodies of NEDD4 (cat. no. 5344; 1:50; Cell Signaling Technology, Inc.) and APEX1 (cat. no. 10203-1-AP; 1:100; ProteinTech Group, Inc.) were used for the co-immunoprecipitation assay and anti-rabbit IgG antibody (cat. no. ab205718, 1:500; Abcam) served as a negative control. The cells were separated, washed twice with PBS and proteins were extracted with RIPA lysis buffer and the supernatant was collected after centrifugation at 13,000 x g for 10 min at 4˚C. The supernatant of cell lysate (500 μg) was incubated with anti-NEDD4 and anti-APEX1 at 4˚C for 24 h. Then, 50 μg protein A magnetic beads were added for capturing the complexes of NEDD4 and APEX1. After the IP reaction, agarose beads were centrifuged at 1,000 x g for 3 min at 4˚C to the bottom of the tube. The supernatant was then carefully absorbed and the agarose beads were washed three times with 1 ml lysis buffer. A total of 15 μl 2X SDS sample buffer was finally added for boiling at 100˚C for 5 min. Afterwards, the collected complexes

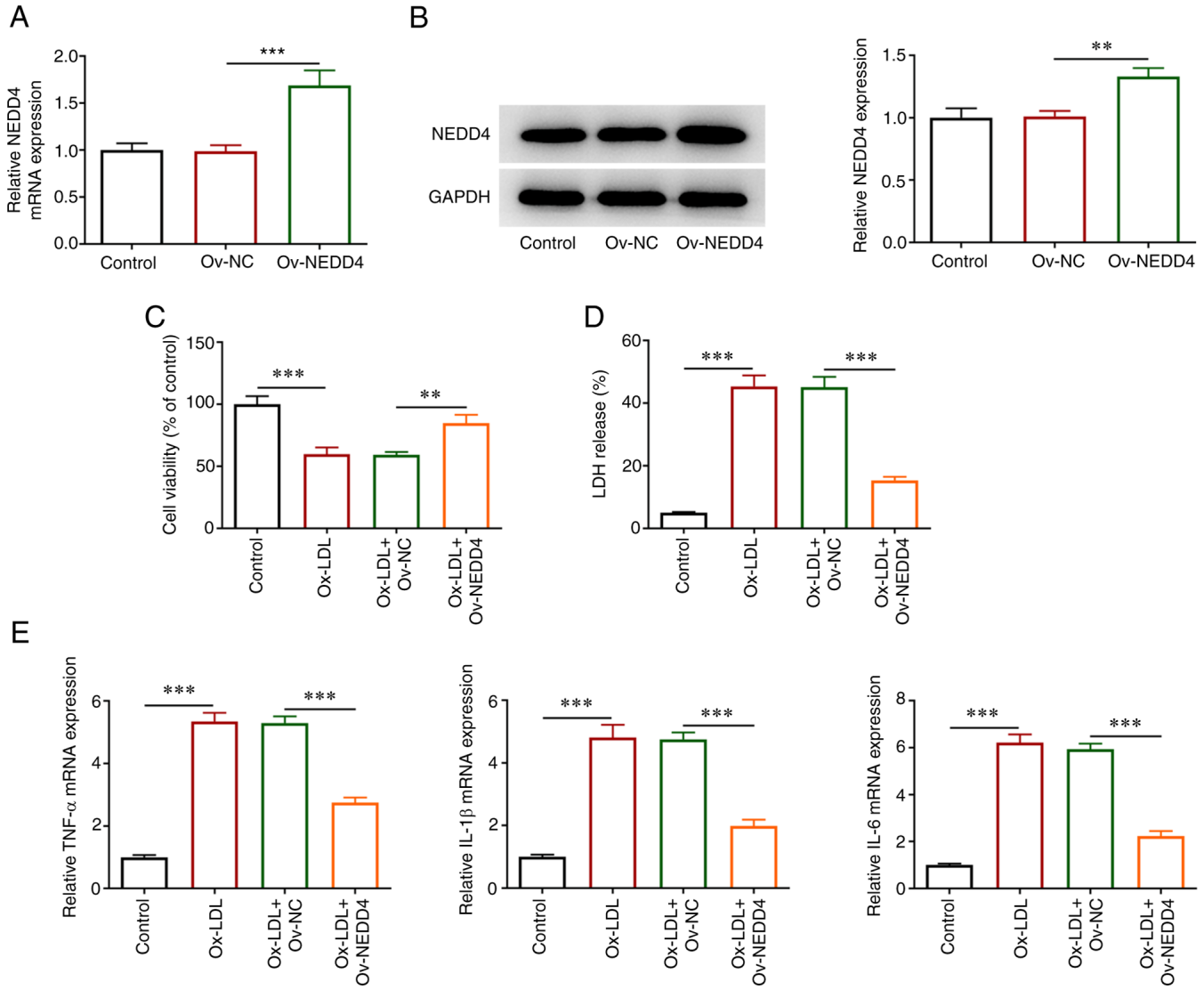

Figure 2. NEDD4 overexpression attenuates ox-LDL-induced cellular damage and the release of inflammatory factors. Detection of NEDD4 expression was performed by (A) RT-qPCR and (B) western blotting. (C) The measurement of cellular damage of HUVECs treated by ox-LDL was conducted using CCK8. (D) The determination of cytotoxic damage was performed by an LDH kit. (E) Detection of TNF-α, IL-1β and IL-6 expression was by RT-qPCR. **P<0.01 and ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; ox-LDL, oxidized low density lipoprotein; RT-qPCR, reverse transcription-quantitative PCR; HUVECs, human umbilical vein endothelial cells; LDH, lactate dehydrogenase.

were subjected to western blotting. Finally, an ECL reagent (Vazyme Biotech Co., Ltd.) was adopted to observe the immunoreactive bands.

Statistical analysis. Data are given as the mean ± SD and were analyzed by IBM SPSS Statistics 25 (IBM Corp.). Student t-test along with one-way ANOVA followed by Tukey's post hoc test was for comparisons among two or more means. P<0.05 was considered to indicate a statistically significant difference.

Results

NEDD4 expression is declined in ox-LDL-induced endothelial cells. To validate the effects of NEDD4, CCK-8 was used to judge cell activity. RT-qPCR and western blotting were performed to verify NEDD4 expression in ox-LDL-exposed HUVECs. Fig. 1A shows a sharp fall in the viability of HUVECs with increasing concentrations of ox-LDL. Additionally, Fig. 1B and C showed that there was a steep drop in NEDD4 expression relative to the control group. The above results suggested that ox-LDL exerted a greater effect on the viability of HUVECs and that NEDD4 expression was remarkably reduced by ox-LDL induction.

NEDD4 overexpression attenuates ox-LDL-induced cellular damage and release of inflammatory factors. To understand the state of HUVECs induced by ox-LDL, overexpression plasmids of NEDD4 were transfected. RT-qPCR, western blotting, CCK-8 and LDH kits were applied for the evaluation of cellular damage and the release of inflammatory factors. As shown in Fig. 2A and B, NEDD4 expression was markedly elevated in contrast to the negative control group. In addition, it can be observed in Fig. 2C that the reduced viability of HUVECs imposed by ox-LDL was conspicuously restored following the overexpression of NEDD4. Cell cytotoxicity was decreased

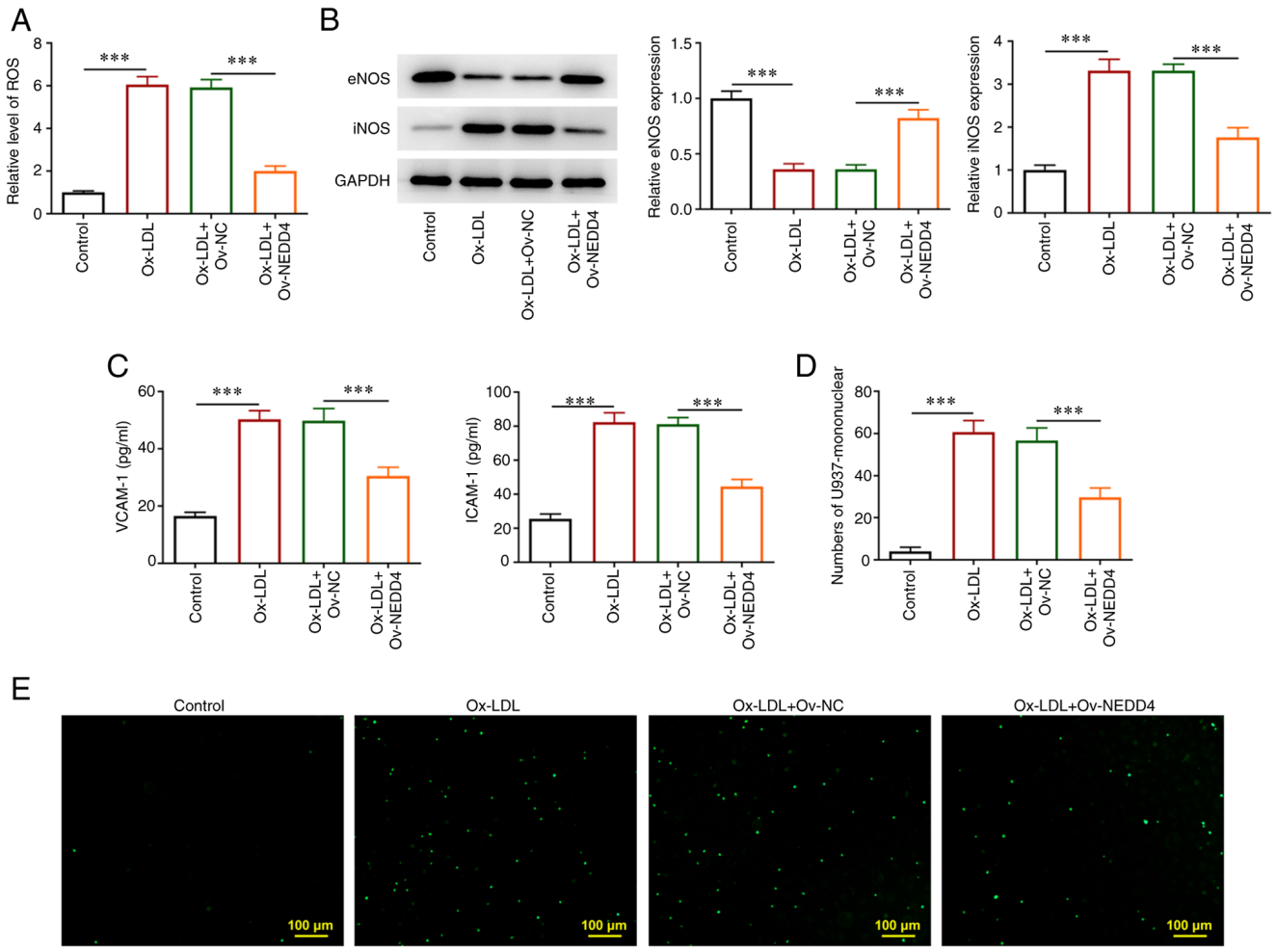

Figure 3. NEDD4 overexpression ameliorates endothelial cell dysfunction of HUVECs induced by ox-LDL. (A) A ROS kit was used to test ROS level in HUVECs treated by ox-LDL. (B) Western blotting was employed to examine eNOS and iNOS expression. (C) ELISA assay was used to detect the expression of VCAM-1 and ICAM-1. (D and E) Adherent cell assay was used to measure the number of endothelial cells adhering to U937 monocytes. ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low density lipoprotein; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule.

(Fig. 2D). Moreover, Fig. 2E shows that TNF-α, IL-1β andIL-6 levels were decreased by contrast with the negative control group. Overall, these results indicated that overexpression of NEDD4 was an efficient way to attenuate cellular damage and inflammatory effects of HUVECs induced by ox-LDL.

NEDD4 overexpression ameliorates endothelial cell dysfunction of HUVECs induced by ox-LDL. ROS content was estimated by corresponding kit. The levels of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), VCAM-1 and ICAM-1 in addition to the number of endothelial cells of U937 monocytes were detected respectively. As seen in Fig. 3A, overexpression of NEDD4 was found to lead to a marked drop in ROS generation. Conversely, Fig. 3B revealed that NEDD4 overexpression promoted eNOS generation but reduced iNOS production. Additionally, the rapid decrease in the levels of adhesion molecules VCAM-1 and ICAM-1 in atherosclerotic lesions in response to NEDD4 overexpression (Fig. 3C). Fig. 3D and E show that the stained cells and the number of endothelial cells that adhered to U937 monocytes were reduced. Together, these results provided important insights that NEDD4 overexpression alleviated inflammatory response and endothelial cell dysfunction of HUVECs induced by ox-LDL.

NEDD4 binds to APEX1 and its overexpression promotes the expression of APEX1. To determine the affinity of NEDD4 with APEX1, RT-qPCR and western blotting were used to examine the expression of APEX1 with or without NEDD4 overexpression. Co-IP assay was used to test whether NEDD4 and APEX1 were combined in HUVECs. There was a steep fall in the expression of APEX1 in ox-LDL-induced HUVECs (Fig. 4A and B). As shown in Fig. 4C and D, NEDD4 protein could directly bind with APEX1 protein in HUVECs. The expression of APEX1 clearly declined in HUVECs following ox-LDL induction, which was increased after NEDD4 overexpression (Fig. 4E and F). The above data showed that NEDD4 could bind to APEX1 and its overexpression was able to promote APEX1 expression.

NEDD4 attenuates cellular damage and release of inflammatory factors in ox-LDL-induced HUVECs via regulating APEX1 expression. To understand the role of NEDD4 and APEX1

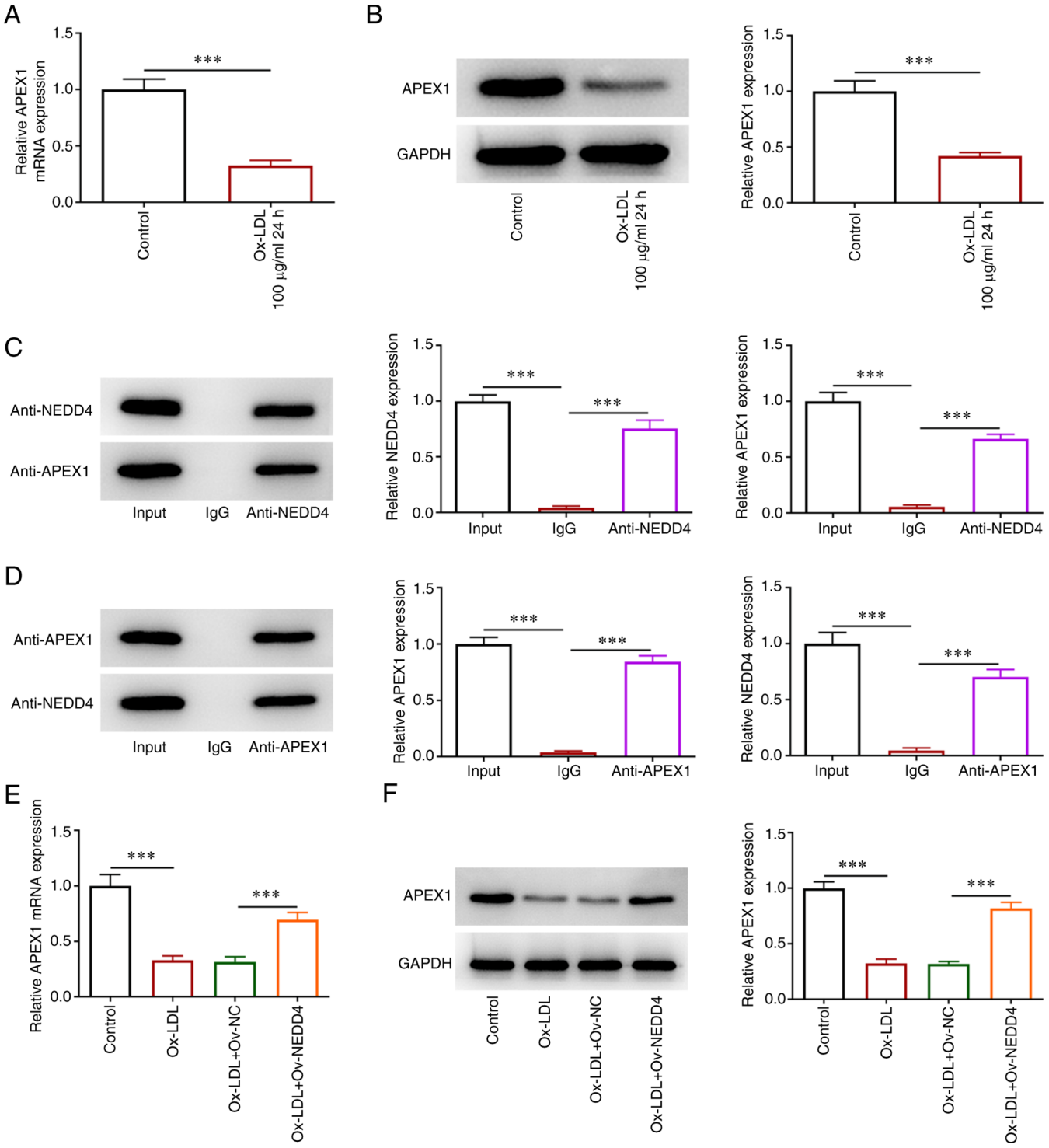

Figure 4. NEDD4 binds to APEX1 and its overexpression promotes the expression of APEX1. Expression of APEX1 in ox-LDL-induced HUVECs was detected by (A) RT-qPCR and (B) western blotting. (C and D) That NEDD4 bound to APEX1 in HUVEC cells was demonstrated by co-immunoprecipitation assay. Expression of APEX1 was determined after overexpression of NEDD4 by (E) RT-qPCR and (F) western blotting. ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; APEX1, apurinic/apyrimidinic endodeoxyribonuclease 1; ox-LDL, oxidized low density lipoprotein; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative PCR.

in the viability injury of HUVECs, the interference plasmids siRNA-APEX1-1 and siRNA-APEX1-2 for APEX1 were constructed. The expression of APEX1 in the siRNA-APEX1-1 group was the lowest among all groups and siRNA-APEX1-1 was selected to perform subsequent experiments (Fig. 5A and B). CCK-8 assay (Fig. 5C) showed that simultaneous NEDD4 elevation and APEX1 absence suppressed the viability of HUVECs following ox-LDL exposure. Using an LDH kit to assess cytotoxic injury, an apparent increase in the cell cytotoxic injury was found (Fig. 5D). TNF-α, IL-1β, IL-6 expression were also fortified relative to the negative group (Fig. 5E). In brief, the results revealed that NEDD4 mitigated cell viability damage and the release of inflammatory factors under the induction of ox-LDL by regulating APEX1 expression.

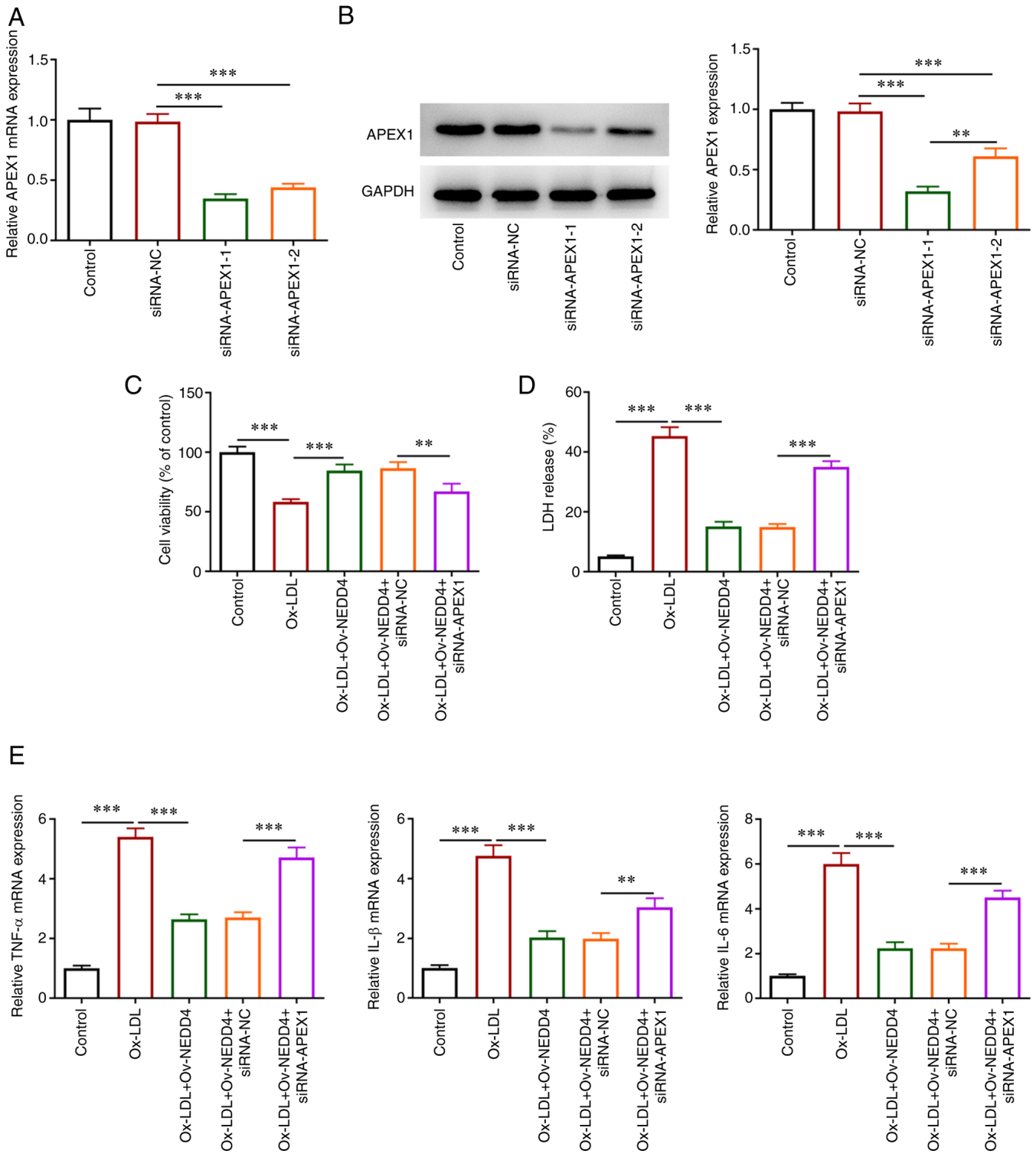

Figure 5. NEDD4 attenuates cellular damage and release of inflammatory factors in ox-LDL-induced HUVECs via regulating APEX1 expression. (A) RT-qPCR and (B) western blotting were employed to detect the interference level of APEX1 following the transfection of siRNA-APEX1-1 and siRNA-APEX1-2. (C) CCK-8 was used to test cellular activity damage. (D) An LDH kit was used to measure cytotoxic damage under the treatment of ox-LDL, overexpression of NEDD4 and interference with APEX1. (E) Detection of TNF-α, IL-1β and IL-6 expression was conducted by RT-qPCR. **P<0.01 and ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; ox-LDL, oxidized low density lipoprotein; HUVECs, human umbilical vein endothelial cells; APEX1, apurinic/apyrimidinic endodeoxyribonuclease 1; RT-qPCR, reverse transcription-quantitative PCR; si, short interfering; LDH, lactate dehydrogenase.

NEDD4 ameliorates ox-LDL-induced endothelial dysfunction by regulating APEX1 expression. To understand the effect of NEDD4 on the endothelial dysfunction of HUVECs induced by ox-LDL through the regulation of APEX1 expression, the level of ROS was first examined using the corresponding kit and it was increased after overexpression of NEDD4 and interference of APEX1 (Fig. 6A). Then, under the aforementioned circumstances, a drop of eNOS protein level and a rise in the protein level of iNOS were observed (Fig. 6B). In addition, the results of ELISA showed that NEDD4 overexpression led to a

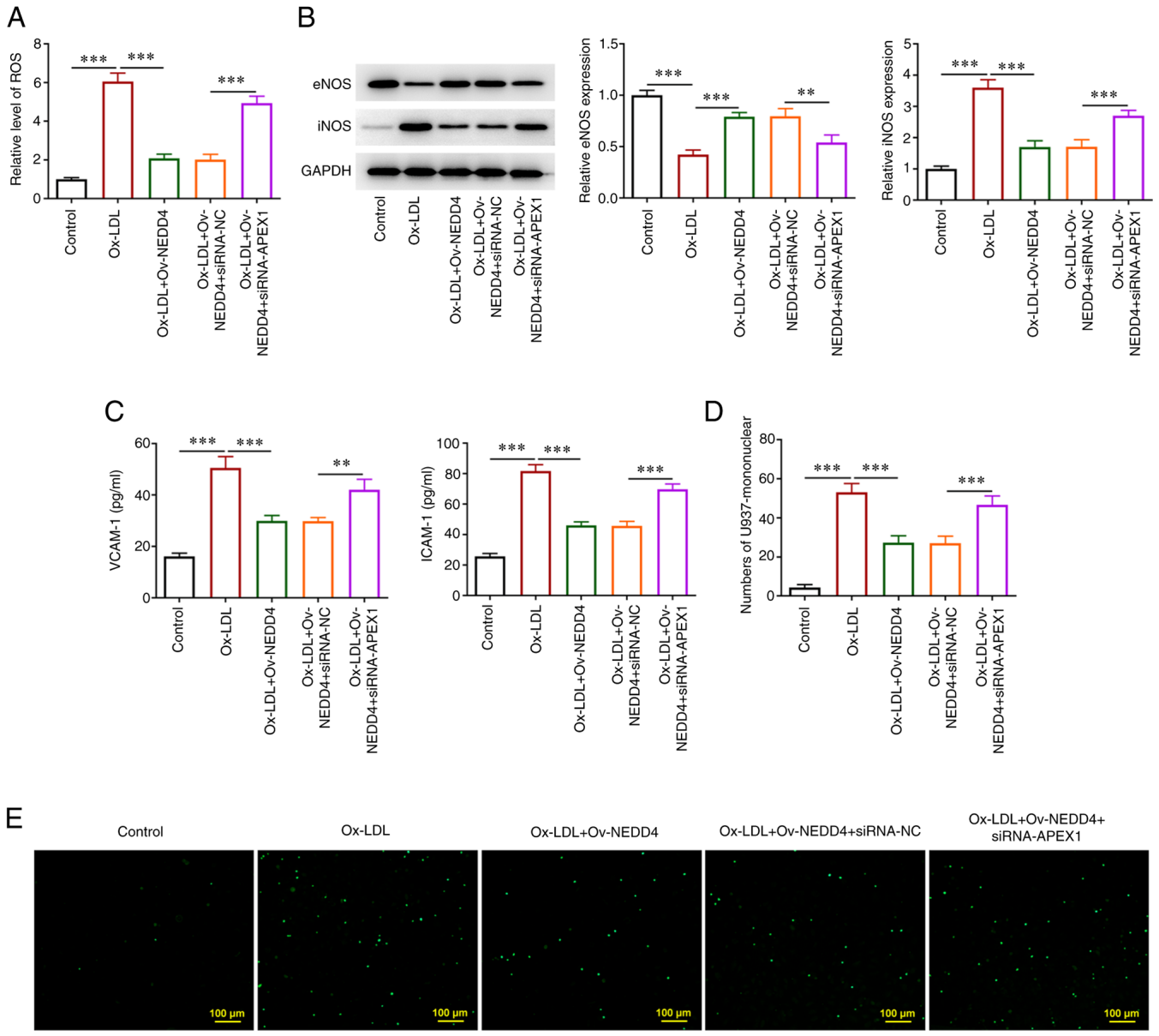

Figure 6. NEDD4 ameliorates ox-LDL-induced endothelial dysfunction by regulating APEX1 expression. (A) A ROS kit was used to evaluate ROS generation under the treatment of ox-LDL, overexpression of NEDD4 and interference with APEX1. (B) Western blotting was adopted to test eNOS and iNOS expression under the same conditions as described above. (C) ELISA was used to identify the expression of VCAM-1 and ICAM-1. (D and E) Adherent cell assay was employed to examine the number of endothelial cells adhering to U937 monocytes. **P<0.01 and ***P<0.001. NEDD4, neuronally expressed developmentally downregulated 4; ox-LDL, oxidized low density lipoprotein; APEX1, apurinic/apyrimidinic endodeoxyribonuclease 1; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule.

significant upregulation of both VCAM-1 and ICAM-1 expression (Fig. 6C). Finally, as shown in Fig. 6D and E, there was an increasing number of stained HUVECs and the number of endothelial cells that adhered to U937 monocytes in the cell adhesion experiment following overexpression of NEDD4 and interference of APEX1. From the above, it can be seen that NEDD4 had a certain effect on migrating ox-LDL-induced endothelial dysfunction by regulating APEX1 expression.

Discussion

AS is a threatening and slowly progressive disease. One of the risk factors for AS is ox-LDL, which can contribute to the production of inflammatory cytokines and result in endothelial dysfunction (3). NEDD4 is a ubiquitin ligase that mediates receptor downregulation, elicits vital activities in AS (14) and has been found to bind to APEX1 in the database. The present study found that NEDD4 overexpression attenuated ox-LDL-induced endothelial cell inflammation and dysfunction and ameliorated these AS-related symptoms by regulating APEX1 expression.

A number of studies about NEDD4 are related to the development of cancers. For example, NEDD4 participates in cell migration in lung cancer through mediating EGFR signaling (20). NEDD4 mediates the reduction of CX43 regulated by fulvestrant in breast cancer cells (21). There are also studies demonstrating the regulatory role of NEDD4 in some of the symptoms of AS. NEDD4 negatively modulates

the activation of non-classical inflammasomes (22). NEDD4 promotes p38α ubiquitination which plays a crucial regulatory role in inflammatory cells (23). A previous study has suggested that NEDD4 can reduce AngII-induced apoptosis in endothelial cells (24). To investigate the role of NEDD4 in endothelial cell dysfunction and inflammation, the present study examined the expression of NEDD4 in ox-LDL-induced HUVECs and found that the overexpression of NEDD4 was capable of enhancing the cell activity, diminishing HUVECs toxicity and cutting down TNF-α, IL-1β and IL-6 expression. These results were consistent with the fact that NEDD4 poses an important role in reducing endothelial dysfunction and inflammation.

The imbalance between reactive ROS and the antioxidant defense system is a major cause of endothelial dysfunction, leading to vascular damage in metabolic and atherosclerotic diseases (25). NO is an anti-atherosclerotic molecule that reduces the inflammatory response of tissues (26). iNOS and eNOS are abundant isoforms expressed in endothelium. eNOS uncoupling is one of the important mechanisms of AS. eNOS decoupling reduces the production of NO and its protective effect on blood vessels (27). Previous experiments have shown that eNOS gene knockout or its inhibitor can accelerate the formation of AS in experimental animals (27). In addition, eNOS is uncoupled to produce superoxide, which increases the oxidative pressure and promotes the formation of AS (28). ROS levels are reduced by eNOS inhibition (29). Under normal circumstances, iNOS is not expressed. However, under some stimuli, iNOS can be regulated through overexpression, transcription and translation and then regulates the synthesis of NO and finally participates in inflammation and injury repair (30). ROS is abundantly produced by iNOS (31). Moreover, macrophages which can be differentiated from U973 monocytes can produce ROS during phagocytosis of foreign particles (32). The inhibition of ROS results in the reduction of U937-HUVECs adhesion (33). In addition, adhesion molecules VCAM-1 and ICAM-2 mediate the migration of cells between the bloodstream and inflamed tissues (34). Moreover, when atherosclerotic injury occurs, endothelial cells release adhesion factors and attract monocytes to accumulate, which can reflect cell adhesion (35), and U937 is a human monocyte. U937 cells were used to detect the changes of HUVEC adhesion ability in the present study (36). It was found that NEDD4 overexpression led to a decrease in the levels of ROS, iNOS, VCAM-1, ICAM-2 and the number of U937-HUVEC adhesion cells, but an increase in the level of eNOS. Consequently, NEDD4 elevation eased ox-LDL-evoked endothelial cell dysfunction.

APEX1 is a multifunctional protein which is related to cancers and cardiovascular diseases (37). APEX1 mediates redox function against vascular calcification which plays a role in the pathogenesis of AS and chronic kidney disease (38). In the present study, the combination of NEDD4 and APEX1 was confirmed, which corresponded to BioGRID database. Subsequently, an interference plasmid for APEX1 was constructed and it was found that NEDD4 promoted APEX1 expression and attenuated ox-LDL-induced inflammation and endothelial dysfunction by regulating APEX1 expression.

Taken together, in the present study, increasing doses of ox-LDL were used to treat HUVECs to simulate the inflammatory environment. NEDD4 and APEX1 expression as well as the influence on ox-LDL-induced endothelial cell inflammation and dysfunction were examined under different conditions. Experimental results showed that NEDD4 could reduce ox-LDL-induced endothelial cell dysfunction and inflammation by promoting the expression of APEX1. These findings are of great value for understanding the underlying mechanism of AS.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HX and WZ conceived and designed the study. LT and QQ performed the experiments. HX and WZ analyzed the experimental data. HX and LT wrote and revised the manuscript. HX and WZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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