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Sirtuin 1 Prevents Arterial Endothelial Cell Injury Triggered by Oxidized Low-density Lipoprotein via Modulating the TXNIP/NLRP3 Signaling Pathway

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KEYWORDS Artery. Damage. Endothelial Cells. Expression. Low-Density Lipoprotein. Signaling Pathway

ABSTRACT The researchers aimed to explore the protective mechanism of sirtuin 1 (Sirt1) against arterial endothelial cell injury triggered by oxidized low-density lipoprotein (Ox-LDL) via modulating the TXNIP/NLRP3 signaling pathway. Lentiviral infection or siRNA transfection was employed to establish human umbilical vein endothelial cells (HUVECs) presenting stably overexpressed Sirt1 or with interfered Sirt1 expression. Compared with control cells, Ox-LDL-treated HUVECs with overexpressed Sirt1 had significantly enhanced proliferative activity and decreased apoptosis rate, while HUVECs with interfered Sirt1 expression had significantly weakened proliferative activity and increased apoptosis rate. In comparison with control cells, HUVECs with overexpressed Sirt1 had significantly decreased TXNIP and NLRP3 protein expressions ($P<0.05$), whereas HUVECs with interfered Sirt1 expression had significantly increased expressions ($P<0.05$). Adding SRI-37330 significantly elevated the expression level of Sirt1 in cells. In the case of Ox-LDL-induced damage to HUVECs, Sirt1 is capable of safeguarding vascular endothelial cell functions by suppressing the activity of the TXNIP/NLRP3 signaling pathway.

INTRODUCTION

As a chronic inflammatory disease based on damage to arterial endothelial cells, atherosclerosis (AS) is one of the main disabling and fatal cardiovascular diseases worldwide (Peng et al. 2020). Arterial endothelial cells are in direct contact with the blood and play key roles in maintaining the vascular function and resisting inflammation and oxidative stress. However, they can be damaged by various factors such as hyperlipidemia, hyperglycemia and hypertension, thereby facilitating AS occurrence and progression (Gao et al. 2020). The interaction between arterial endothelial cells and oxidized low-density lipoprotein (Ox-LDL) poses crucial impacts on AS advancement (Catar et al. 2022). Ox-LDL contains abundant oxidized lipids, oxidized phospholipids and other substances, which can activate endothelial cells and deprive them of normal physiological functions, thus promoting vascular inflammation and oxidative stress response, increasing the expressions of cell adhesion molecules, and affecting vascular permeability (Chen et al. 2019; Gao et al. 2021). Several signaling pathways have been identified in recent decades with participation in the process of Ox-LDL-induced damage to arterial endothelial cells, including the thioredoxin-interacting protein (TXNIP)/nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) signaling pathway (Guo et al. 2021).

TXNIP, a reactive oxygen species (ROS)-sensitive protein, is able to promote the generation of intracellular oxidative stress through the suppression of glutathione peroxidase plus reduced glutathione from the aspect of activity (Choi and Park 2023). In the case of oxidative stress, intracellular TXNIP further increases and significantly activates NLRP3 inflammasomes, resulting in inflammation

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and cellular damage (Li et al. 2019). Sirtuin 1 (Sirt1) is a deacetylase dependent of nicotinamide adenine dinucleotide, which participates in modulating endothelial cell functions and protecting the vascular health (He et al. 2019). Sirt1 modifies manifold substrate proteins through deacetylation and regulates various biological processes such as oxidative stress, inflammatory responses and metabolic processes. In addition, Sirt1 is capable of effectively modulating the TXNIP/NLRP3 signaling pathway to inhibit the progression of AS (Lian et al. 2023).

Objectives

This study was performed to explore whether Sirt1 can repress Ox-LDL-induced damage to arterial endothelial cells by modulating the TXNIP/NLRP3 signaling pathway, aiming to provide a theoretical basis and innovative thoughts for preventing and curing AS.

MATERIAL AND METHODS

Reagents and Materials

Gibco (USA) supplied penicillin/streptomycin, fetal bovine serum, and Dulbecco's modified Eagle medium (DMEM). Suzhou Ribo Life Science Co., Ltd. (China) offered the cell counting kit-8 (CCK-8) assay kit. The bicinchoninic acid protein assay kit and RIPA cell lysis buffer were provided by Shanghai Beyotime Biotechnology Co., Ltd. (China). TRIzol reagent was purchased together with sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel preparation kit from Beijing Solarbio Science & Technology Co., Ltd. (China). TaKaRa (Japan) was the supplier of real-time polymerase chain reaction (RT-PCR) kit plus reverse transcription kit. Lentivirus overexpressing Sirt1 and its control (NC1), as well as small interfering ribonucleic acid (siRNA) specifically interfering with Sirt1 expression and its control (NC2), sourced from Guangzhou RiboBio Co., Ltd. (China). Primary antibodies against Caspase-3 (#9662), Sirt1 (#8469), B-cell lymphoma-2 (Bcl-2) (#15071), interleukin-1 (IL-1) (#50794), Caspase-9 (#9508), Bcl-2-associated X protein (Bax) (#41162), tumor necrosis factor-α (TNF-α) (#11948), NLRP3 (#13158), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#92310), TXNIP (#14715), and IL-6 (#12912) were provided by Cell Signaling Technology (USA).

Construction of Cell Model

Complete DMEM was employed to inoculate human umbilical vein endothelial cells (HUVECs) for 37°C culture using an incubator under 5 percent CO_2. Then they were passaged at a ratio of 1:3 when the confluence reached 90 percent. Afterwards, the third passage (P3) cells were collected for further cell experiments. The complete medium was supplemented with pEGFP-N1-GFP-Sirt1-containing lentiviral suspension for 24 h of 37°C incubation. Then a fresh complete medium was added following the removal of the medium with viral particles. Forty-eight hours later, cells with overexpressed Sirt1 were successfully constructed based on Western blotting together with RT-quantitative PCR (RT-qPCR). Moreover, as per the manufacturer's instructions, siRNA-Sirt1 and si-NC2 were transfected into the cells for 24 h using Lipofectamine 2000 kit supplied by Thermo Fisher Scientific (USA), and the medium was replaced with a normal one.

CCK-8 Assay for Detecting Cell Viability

The cell viability was determined by means of the CCK-8 assay kit. In brief, 5×10^3 cells were inoculated into 96-well plates for 24-h culture. Next, the CCK-8 reagent (10 μL) was supplemented for 1 h of incubation at 37°C in a cell culture incubator. Afterwards, a microplate reader purchased from Thermo Fisher Scientific (USA) was utilized to obtain the optical density at 450 nm.

Ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Assay

The 96-well plates were selected for overnight inoculation of cells in the logarithmic growth phase (4×10^5/well in density). Then all wells were added with EdU solution (100 μL) diluted (1:1000) by culture medium, followed by 2 h of incubation. Subsequently, phosphate-buffered saline was used to wash the cells by three times, added Apollo staining solution, subjected to 30 min of incubation, and washed for three times in phosphate-buffered saline. Subsequently, the cells were added DAPI

and methanol fixative sequentially. Finally, a fluorescence microscope (200×) (Olympus, Japan) was applied to photograph the cells from at least 8 randomly selected fields of view.

Enzyme-linked Immunosorbent Assay (ELISA)

Malondialdehyde (MDA), ROS, and superoxide dismutase (SOD) were measured for their contents in cells using an ELISA kit (Thermo Fisher Scientific, USA). Specifically, the kit plates were supplemented with the cell culture supernatant (100 μL), followed by incubation and washing. Next, incubation was conducted with diluted antibodies against ROS, MDA and SOD (1:100). Following washing, every well was added with horseradish peroxidase-labeled streptavidin (1:100 dilution, 100 μL) for incubation, followed by washing. Then the wells were incubated in a dark room with color-generating substrate TMB added in a volume of 100 μL for 10 min, and stop buffer was added to each well in a volume of 100 μL. At last, the wavelengths at 450 nm and 570 nm were examined by the microplate reader to read the optical densities.

Apoptosis Assay by Flow Cytometry

A total of 2×10^6 HUVECs were inoculated in 6-well plates, digested with 0.25 percent trypsin and collected. Then the annexin V-fluorescein isothiocyanate apoptosis assay kit (Cell Signaling Technology, USA) was used for apoptosis analysis. In brief, the cells underwent 15 min of room-temperature staining using annexin V-fluorescein isothiocyanate and PI, and then a flow cytometer (Thermo Fisher Scientific, USA) was utilized to test about 5,000 cells for their apoptosis rate.

RT-qPCR

An RNA extraction kit, together with PrimeScript RT Master Mix and SYBR Premix Ex Taq kit (TaKaRa, Japan), was adopted for extraction of total RNA from cells and reverse transcription into cDNA, respectively, according to the manufacturer's instructions, prior to amplification. With the internal reference gene set as GAPDH, the calculation of relative expression of mRNA was implemented via the $2^{-\Delta\Delta Ct}$ method. Table 1 presents the sequences of gene primers.

Western Blotting

The lysis product obtained from lysis buffer-treated cells underwent 15 min of 4°C centrifugation at 14,000 rpm. Next, the bicinchoninic acid protein assay kit was employed to quantify the proteins in the harvested supernatant. The extracted proteins were translocated to a polyvinylidene fluoride membrane after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then skimmed milk (5%) was added to soak the membrane for 2 h to obstruct non-specific binding sites, and primary antibodies against Sirt1 (diluted at 1:1000), Bax (1:1000 dilution), Caspase-3 (dilution ratio: 1:1000), Caspase-9 (1:1000 dilution), Bcl-2 (dilution ratio: 1:1000), IL-1 (1:1000 dilution), IL-6 (diluted at 1:1000), TNF-α (dilution ratio: 1:1000), TXNIP (1:1000 dilution), NLRP3 (diluted at 1:1000), and GAPDH (diluted at 1:1000) were utilized for incubation at 4°C overnight. Subsequently, corresponding secondary antibodies conjugated with peroxidase were supplemented for 2-h incubation after membrane washing. Finally, an enhanced chemiluminescence detection system (Bio-Rad, USA) was adopted for band examination, where GAPDH was used as the internal reference control.

Statistical Analysis

All assays were implemented independently in triplicate at least. SPSS 20.0 software (IBM Inc., USA) was employed to accomplish statistical

Table 1. Primer sequences

Gene	*Upstream sequence*	*Downstream sequence*
Sirt1	5'CCAGCAGTATGAGAAACCM-3'	5'-TGACGACCGGCATAAAGT-3'
IL-1	5'-CCGATCCTTTGCCGCCTC-3'	5'-GGTCTCATCTAAGATGGT-3'
IL-6	5'-ATAGGTCTCAACGACTAG-3'	5'-ATGACACTATTTAGGTAG-3'
TNF-α	5'-CAACAATGAGCGGAATCC-3'	5'-TGTACGACAACCAAGGGT-3'
TXNIP	5'-TTGGAGGTCGAGCACCC-3'	5'-GGTTCAAGGATGATAAC-3'
GAPDH	5'- AACCATCTCGCAAATAA-3'	5'-ACGCACAGAATCTAGCG-3'

analysis. Mean ± standard deviation was utilized to describe the measurement data, with one-way analysis of variance for comparison among multiple groups and LSD-*t* test for inter-group comparison. $P<0.05$ indicated a difference of statistical significance.

RESULTS

Expression of Sirt1 in HUVECs and Cell Model Construction

Sirt1 significantly dropped in HUVECs at the protein and mRNA expression level after Ox-LDL treatment ($P<0.05$, Fig. 1A). To explore how Sirt1 affects the damage to vascular endothelial cells, HUVECs with overexpressed Sirt1 and those with siRNA-interfered Sirt1 expression were constructed, and the construction was proved successful through Western blotting plus RT-qPCR ($P<0.05$, Fig. 1B and C).

Effect of Sirt1 Expression on Proliferative Activity of HUVECs

As revealed by the CCK-8 assay, the proliferative activity of Ox-LDL-treated HUVECs was sig-

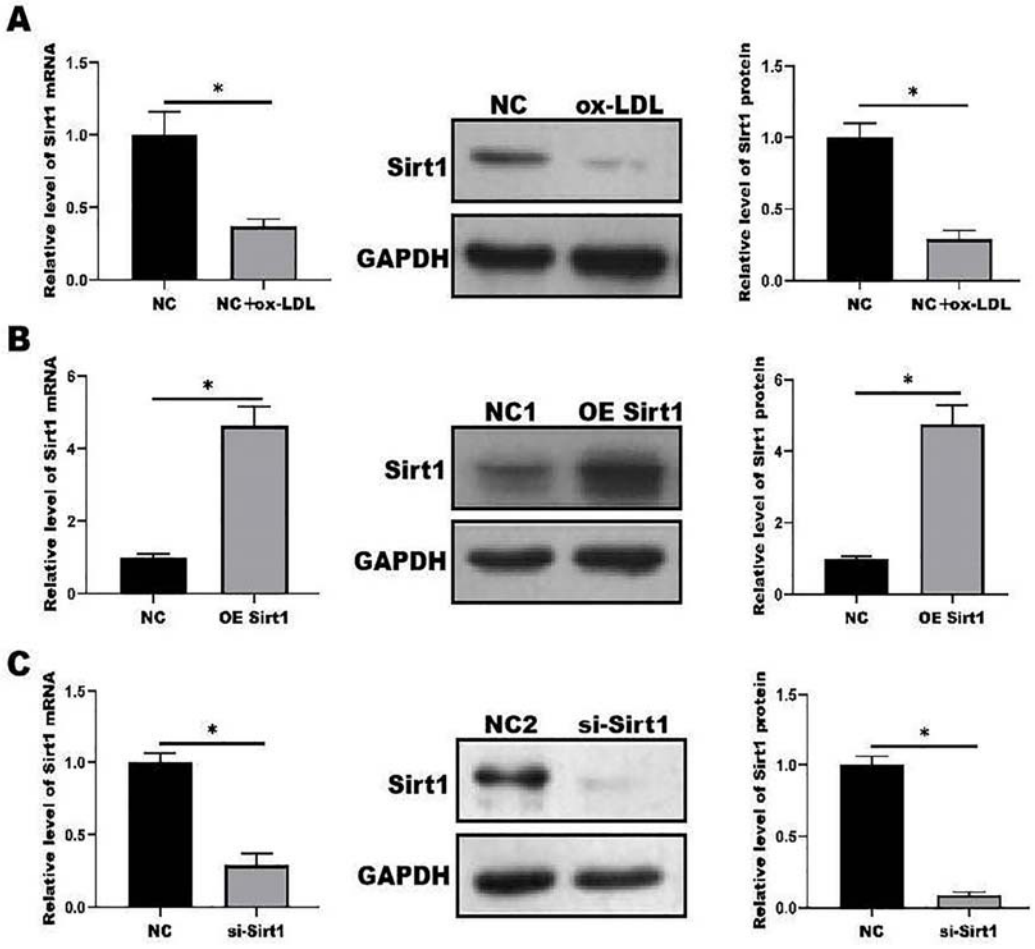

Fig. 1. Expression of Sirt1 in HUVECs and cell model construction

nificantly decreased at 24 h and 48 h. By contrast to corresponding control cells, Ox-LDL-treated HUVECs with overexpressed Sirt1 had significantly enhanced proliferative activity, while HUVECs with interfered Sirt1 expression had significantly weakened proliferative activity ($P<0.05$, Fig. 2A). The EdU cell proliferation assay yielded coherent results with the CCK-8 assay (Fig. 2B).

Effect of Sirt1 Expression on HUVEC Apoptosis

According to flow cytometry, the apoptosis rate of HUVECs significantly rose after Ox-LDL treatment ($P<0.05$). In comparison with corresponding control cells, HUVECs with overexpressed Sirt1 had a significantly reduced apoptosis rate ($P<0.05$), whereas HUVECs with interfered Sirt1 expression had an elevated apoptosis rate ($P<0.05$) (Fig. 3A). Bax, Caspase-3, and Caspase-9 as pro-apoptotic proteins presented significantly raised expression levels ($P<0.05$), whereas Bcl-2 as an anti-apoptotic protein exhibited a significantly reduced expression level ($P<0.05$) in Ox-LDL-processed HUVECs. Moreover, Bax, Caspase-3, and Caspase-9 dropped significantly, but Bcl-2 rose at the expression level in HUVECs with overexpressed Sirt1 contrasted

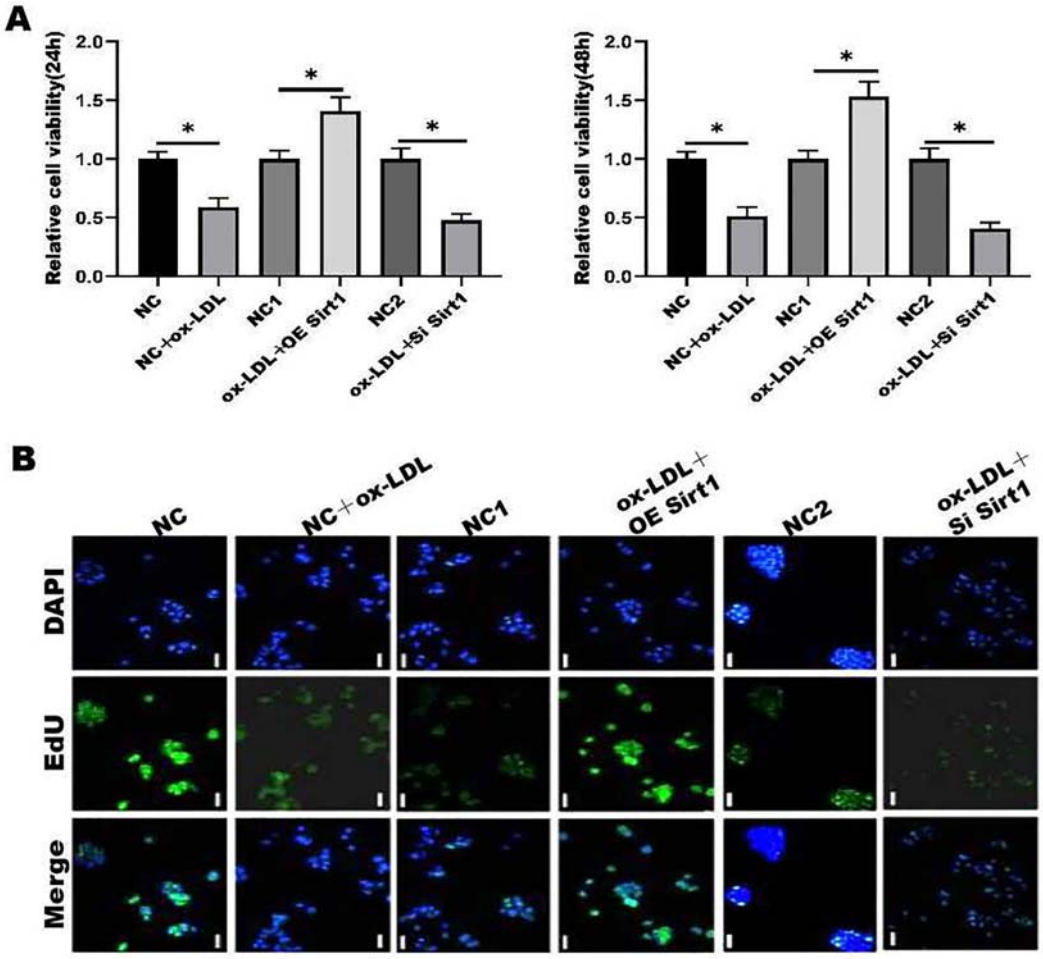

Fig. 2. Role of Sirt1 expression in affecting proliferative activity of HUVECs

with corresponding control cells (P<0.05). Opposite trends were observed in HUVECs with interfered Sirt1 expression (P<0.05) (Fig. 3B).

Role of Sirt1 Expression in influencing Inflammatory Cytokines together with Oxidative Stress Response in HUVECs

IL-1, IL-6, and TNF-α in HUVECs had significantly elevated expression levels after Ox-LDL treatment (P<0.05). These levels significantly declined in HUVECs with overexpressed Sirt1 (P<0.05) and rose in HUVECs with interfered Sirt1 expression (P<0.05) in comparison to those in corresponding control cells (Fig. 4A). These findings suggested an obvious protective effect of Sirt1 on Ox-LDL-mediated inflammatory injury of vascular endothelial cells.

The ELISA results revealed that Ox-LDL-treated HUVECs had significantly increased contents of ROS and MDA (P<0.05) as well as decreased SOD concentration (P<0.05). Compared with control cells, HUVECs with overexpressed Sirt1 had significantly incremented SOD content (P<0.05) along with lowered contents of ROS and MDA (P<0.05), while HUVECs with interfered Sirt1 expression showed opposite trends (Fig. 4B).

TXNIP/NLRP3 Signaling Pathway Expression in HUVECs processed by Ox-LDL

After processing with Ox-LDL, HUVECs presented significant activation of the TXNIP/NLRP3 signaling pathway, with significantly elevated TXNIP protein and NLRP3 protein expression levels (P<0.05). Additionally, in terms of the protein expression level, TXNIP and NLRP3 dropped significantly in HUVECs with overexpressed Sirt1 (P<0.05) and rose in HUVECs with interfered Sirt1 expression (P<0.05) compared with those in control cells, implying the potential implication of Sirt1 in controlling Ox-LDL-mediated damage to vascular endothelial cells through the TXNIP/NLRP3 signaling pathway. For the purpose of exploring whether the TXNIP/NLRP3 signaling pathway has a direct regulatory relationship with Sirt1, SRI-37330,

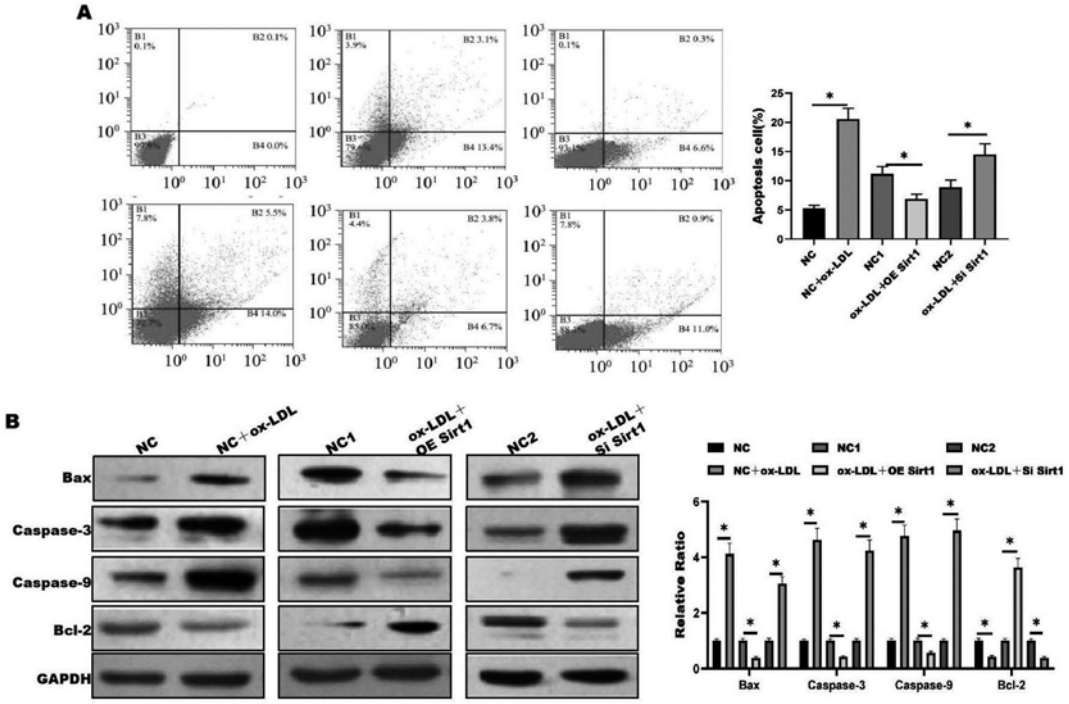

Fig. 3. Function of Sirt1 expression in influencing HUVEC apoptosis

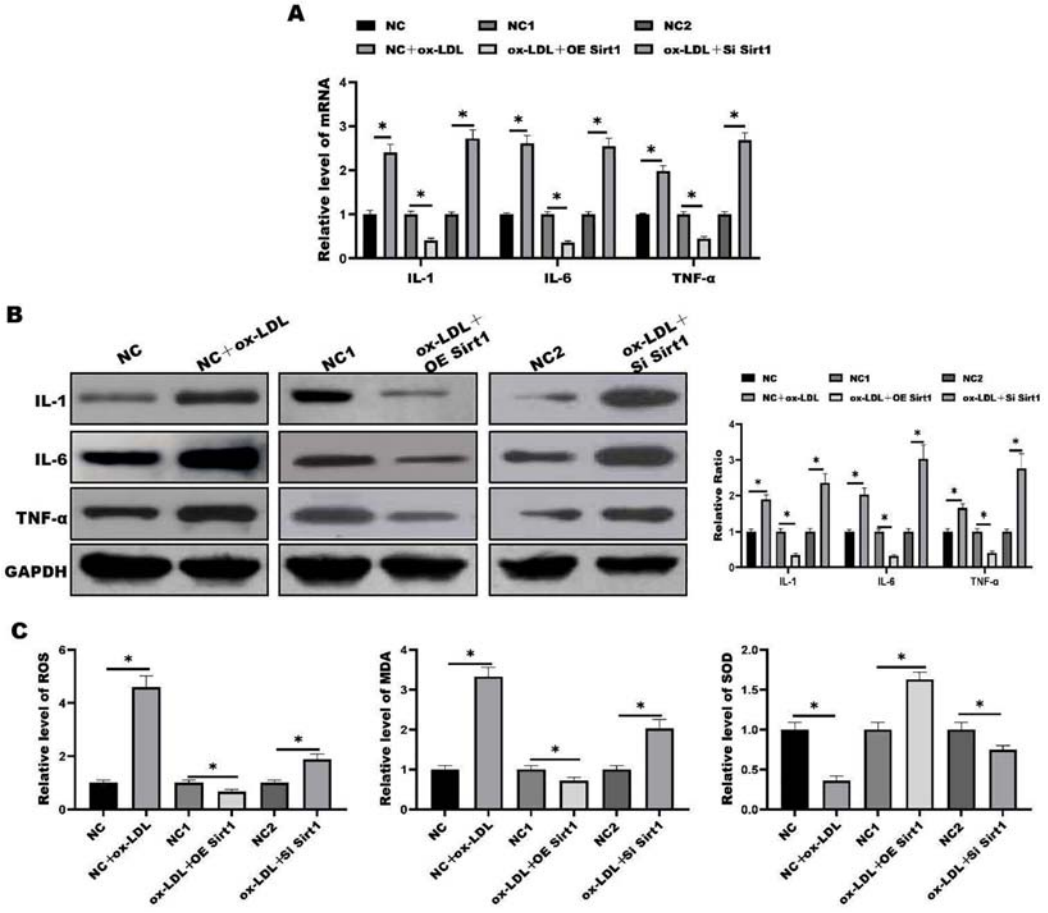

Fig. 4. Effects of Sirt1 expression on inflammatory cytokines and oxidative stress response in HUVECs.

an inhibitor of TXNIP, was used to interfere with the TXNIP/NLRP3 signaling pathway activity. Compared with Ox-LDL treatment, SRI-37330 treatment gave prominent rise to Sirt1 expression level (Fig. 5).

DISCUSSION

Damage to arterial endothelial cells is one of the important early events in AS progression (Mussbacher et al. 2022). Hence, investigating the mechanism of Ox-LDL-induced damage to arterial endothelial cells can help develop new therapeutic strategies for AS. For instance, inflammatory responses and oxidative stress play crucial roles in Ox-LDL-induced damage to endothelial cells (Zheng et al. 2023). Therefore, anti-inflammatory and antioxidant interventions can relieve damage to arterial endothelial cells, thereby improving the prognosis and therapeutic outcomes of AS.

Sirt1 exerts vital regulatory effects on many biological processes such as apoptosis, oxidative stress, inflammatory responses, and cell proliferation (Yang et al. 2022). In this study, arterial endothelial cells processed using Ox-LDL displayed a remarkably lowered Sirt1 expression level. This may be attributed to oxidative stress (Yuan et al. 2020; Wang et al. 2021), inflammatory responses (Sasaki et al. 2020; Karnewar et al. 2022) and endothelial dysfunction (Luo et al. 2019; Zhou et al. 2022). To

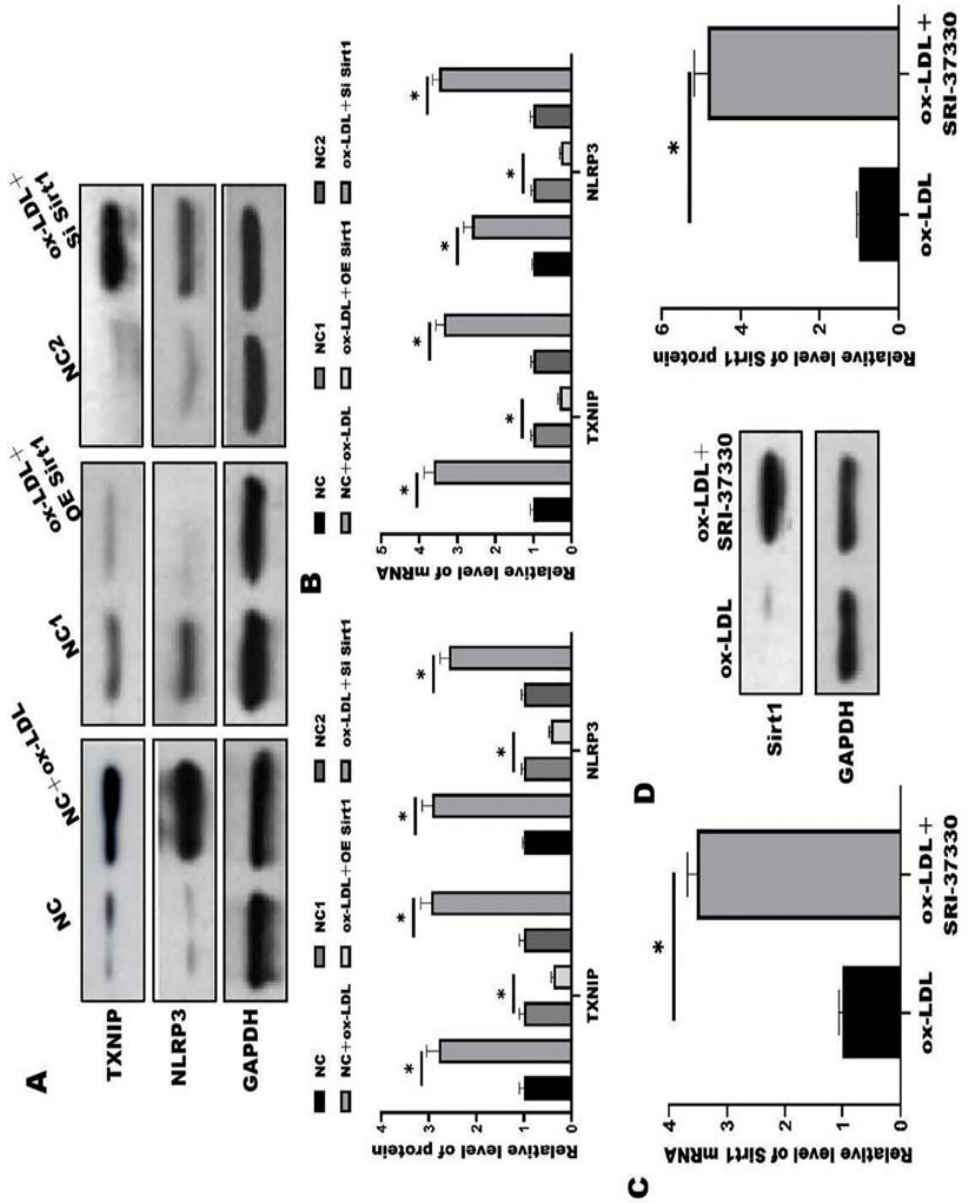

Fig. 5. Expression of TXNIP/NLRP3 signaling pathway in Ox-LDL-treated HUVEC

better explore the role of Sirt1 in vascular endothelial cells, cells with overexpressed Sirt1 and with interfered Sirt1 expression were constructed. The researchers found that overexpressing Sirt1 effectively promoted the propagation but suppressed the apoptosis of endothelial cells, while interfering with Sirt1 expression repressed the multiplication of endothelial cells and facilitated their apoptosis, suggesting that Sirt1 obviously regulated vascular endothelial cells from the aspects of proliferation and apoptosis.

Moreover, Ox-LDL-treated HUVECs exhibited excessive oxidative stress responses, reflected as elevated intracellular ROS and MDA contents and reduced SOD content. However, HUVECs with overexpressed Sirt1 had significantly decreased contents of ROS and MDA and markedly increased SOD, whereas the opposite trends were observed in HUVECs with interfered Sirt1 expression. The results may be ascribed to the following reasons. Sirt1 can inhibit the activity of NADPH oxidase, thereby reducing the production of ROS (Xia et al. 2021). Meanwhile, Sirt1 can regulate mitochondrial biosynthesis to optimize mitochondrial function while attenuating ROS generation. Additionally, Sirt1 is capable of strengthening the expression together with activity of antioxidant enzymes by down-regulating the demethylation modification of antioxidant enzyme genes, thus improving the antioxidant capacity (Raj et al. 2021).

The TXNIP/NLRP3 signaling pathway serves as a key player in the damage to vascular endothelial cells caused by Ox-LDL (Saber et al. 2021). As an intracellular regulatory protein, TXNIP binds reduced glutathione and works as an antioxidant factor by maintaining the intracellular redox balance in normal cases (Yuan et al. 2022). In the case of Ox-LDL-induced damage to vascular endothelial cells, however, TXNIP expression is remarkably elevated. TXNIP forms a complex with NLRP3 to trigger the formation of NLRP3 inflammasomes, which in turn triggers inflammatory responses and initiates pro-apoptotic pathways (Yuan et al. 2022). The activated NLRP3 inflammasomes further activate Caspase-1, giving rise to the production and release of IL-1, IL-6, and other inflammatory cytokines (Hang et al. 2020; Yan et al. 2021). These inflammatory cytokines further induce inflammatory responses and apoptosis of endothelial cells. Similarly, it was uncovered through the present study that significant enhancement of the TXNIP/NLRP3 signaling pathway activity was observed in HUVECs after Ox-LDL treatment, and the expression level of Sirt1 rose evidently after interfering with the activity of the TXNIP/NLRP3 signaling pathway using TXNIP inhibitor SRI-37330, indicating that Sirt1 can modulate the TXNIP/NLRP3 signaling pathway activity in HUVECs.

CONCLUSION

In conclusion, regarding the Ox-LDL-mediated damage to HUVECs, Sirt1 protects the function of vascular endothelial cells by suppressing the activity of the TXNIP/NLRP3 signaling pathway.

RECOMMENDATIONS

This study casts new insights into and exploits a potential target for AS diagnosis and treatment.

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