Original Paper

SIRT1 Regulates CD40 Expression Induced by TNF- α via NF- κ B Pathway in Endothelial Cells

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Key Words

SIRT1 • Cluster of differentiation 40 • Endothelial cells • Tumor necrosis factor- α • Nuclear factor-kappa B • Inflammation • Atherosclerosis

Abstract

Background: Compelling evidence suggests that SIRT1, NAD⁺-dependent class III protein deacetylase, plays an important role in the prevention and treatment of atherosclerosis by counteracting inflammation. Cluster of differentiation 40 (CD40), as a pro-inflammatory cytokine, has been shown to participate in the pathophysiology of atherosclerosis. The relationship between SIRT1 and CD40, however, remained elusive. The present study was thus designed to explore the potential effect of SIRT1 on CD40 expression induced by tumor necrosis factor- α (TNF- α) and to disclose the underlying mechanism in CRL-1730 endothelial cells. **Methods:** mRNA and protein expressions were identified by quantitative real-time PCR and Western blot respectively. Subcellular localization of SIRT1 was detected by immunofluorescence analysis. SIRT1 small-interfering RNA (siRNA) was carried out for mechanism study. **Results:** TNF- α reduced SIRT1 expression and induced CD40 expression in CRL-1730 endothelial cells in a time- and concentration- dependent manner. Pretreatment with resveratrol (a potent SIRT1 activator) inhibited TNF- α -induced CD40 expression, while pretreatment with nicotinamide (class β HDACs inhibitor nicotinamide) or sirtinol (a known SIRT1 inhibitor), especially SIRT1 siRNA significantly augmented TNF- α -induced CD40 expression. The further study indicated that PDTC (NF- κ B inhibitor) pretreatment attenuated TNF- α -induced CD40 expression, and SIRT1 siRNA significantly augmented TNF- α -induced acetylated-NF- κ B p65 (Lys310) expression. **Conclusion:** The present study provides the direct evidence that SIRT1 can inhibit TNF- α -induced CD40 expression in CRL-1730 endothelial cells by deacetylating the RelA/p65 subunit of NF- κ B at lysine 310, which provides new insights into understanding of the anti-inflammatory and anti-atherosclerotic actions of SIRT1.

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Introduction

Sirtuins, the class III histone deacetylases (HDACs), are widely distributed and constitute a recently identified mammalian family of regulatory molecules (Sirtuin 1 to Sirtuin 7) that have been implicated in the regulation of critical cellular processes such as differentiation, proliferation, apoptosis, and senescence [1]. Sirtuin 1 (SIRT1), the best characterized and well-studied among Sirtuins, plays an important role in the regulation inflammation-associated diseases by suppressing the release of pro-inflammatory cytokines [2-5]. Recently, emerging evidence implicates that SIRT1 is a novel target to prevent atherosclerosis [6, 7]. Endothelium-specific over-expression of SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice [8], whereas genetic deletion of SIRT1 in atherosclerotic mice increases the atherosclerotic plaques. It's well known that atherosclerosis is a chronic inflammatory disease [9], and pro-inflammatory cytokines have a profound impact on the development of the atherosclerotic plaque. Cluster of differentiation 40 (CD40), as a pro-inflammatory cytokine, has already been showed to be over-expressed in multiple cell types in atherosclerotic lesion, especially in advanced plaques [10]. The interaction between CD40 and CD40 ligand (CD40L) contributes to the initiation, progression, and complications of atherosclerosis [11-14]. Our previous studies have also shown that CD40 expression was increased in aortas of rabbits by giving high fatty diet [15, 16], and in human umbilical vein endothelial cell (HUVECs) treated with hydrogen peroxide [16]. However there is no direct evidence to demonstrate the relationship between SIRT1 and CD40. Therefore, this study was conducted to explore the possible effect of SIRT1 on TNF- α -induced CD40 expression in CRL-1730 endothelial cells. Furthermore, It's well accepted that the anti-inflammatory properties of SIRT1 are closely related to its negative regulation of nuclear transcription factor (NF- κ B) [17]. Our previous study and other studies have also shown that NF- κ B is involved in CD40 expression in HUVECs [18], in macrophages [19], and in vascular smooth muscle cells [20]. So we also investigated whether NF- κ B pathway was involved in the modulatory effect of SIRT1 on CD40 expression in CRL-1730 endothelial cells.

Materials and Methods

Materials

TNF- α was obtained from PeproTech (Rocky Hill, NJ, USA). Polyclonal anti-CD40 and anti-acetyl-NF- κ B p65 (Lys310) antibodies were obtained from Abcam Inc. (Cambridge, MA, UK). SIRT1 (H-300): sc-15404 is a rabbit polyclonal antibody raised against amino acids 448-747 at the C-terminus of SIRT1 of human origin, and was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- β -actin antibody, goat anti-rabbit horseradish peroxidase-linked second antibody and fluorescein isothiocyanate (FITC)-conjugated second antibody were all purchased from Bioss Biological Technology Co. Ltd. (Beijing, China). Resveratrol (RSV), nicotinamide (NAM), Sirtinol and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 was supplied by Gibco BRL (Grand Island, NY, USA). New-born Calf Serum was purchased from Yin xiang Wei ye Group (Shandong, China).

Cell culture

Human umbilical vein endothelial cell line (CRL-1730) was purchased from the American Type Culture Collection (ATCC). Endothelial cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated new-born calf serum, 1% sodium bicarbonate, and 1% penicillin/streptomycin at 37°C in humidified atmosphere of 5% CO₂. Endothelial cells were plated at an appropriate density according to each experimental scale, in 24- and 6-well plates for immunofluorescence, western blot and quantitative real-time PCR assay. The medium was replaced with serum-free medium for an additional 12 h culture before further treatments, and then stimulated by TNF- α as indicated. In the mechanism experiments, endothelial cells were exposed to TNF- α (10 μ g/L) for 8h after pretreated with the activator or inhibitor for 1 h. Endothelial cells treated with serum-free medium only served as control group.

Small-Interfering RNA

Endothelial cells were seeded into 6-well plates. When grown until 70–80% confluent, the cells were transiently transfected with SIRT1 siRNA (100 nM) or NC siRNA (GenePharma, Shanghai, China) using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After 48 h, the silencing effect of SIRT1 was confirmed by western blot. All assays were performed at least 48 h after RNA transfection.

Immunofluorescence analysis

Immunofluorescence analysis was carried out according to standard methods. Briefly, endothelial cells were plated on cover slips. When grown to 60–80% confluence, endothelial cells were treated with 10 μ g/L TNF- α for 8 h, and then the cells were fixed in 10% formaldehyde-PBS for 30 min. After the cell membrane was fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum, the cells were incubated with polyclonal anti-rabbit SIRT1 antibody (1:50 dilution) at 4°C overnight, and then incubated with the secondary antibody conjugated to FITC (1:50 dilution) for 1 h at room temperature. After the cellular nuclei were stained with DAPI (1:1000 dilution), the slides were observed under fluorescent BX-60 microscope (Olympus, Tokyo, Japan).

Western blot analysis

Endothelial cells were collected, washed with PBS and lysed at 4°C in RIPA buffer with the protease inhibitor cocktail (Roche, Mannheim, Germany) for 15 min. After centrifugation at 12,000 $\times$ g for 10 min, supernatant was separated and stored at –70°C until use. Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, and incubated at 4°C overnight with rabbit polyclonal anti-SIRT1 (1:200 dilution), anti-CD40 (1:100 dilution), anti-acetyl-NF- κ B p65 (Lys310) (1:200 dilution) and anti- β -actin (1:400 dilution) antibodies respectively. β -actin was used as internal standard. Then, the membranes were washed in Tris-Buffered Saline Tween-20 (TBST). A horseradish peroxidase-linked antibody (goat anti-rabbit, 1:5000 dilution) was employed as a secondary antibody. The immunostaining was visualized by the enhanced chemiluminescence (Pierce, Rockford, USA).

Quantitative real-time polymerase chain reaction (PCR) assay

Total RNA was isolated from endothelial cells using TRIzol reagent (Invitrogen, CA, USA). cDNA were synthesized from 2 μ g of total RNA using Revert Aid™ First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. The real-time PCR was performed using the SYBR Green PCR Master Mix (Fermentas, St. Leon-Rot, Germany) on an iCycler IQ™5 thermocycler (Bio-Rad, USA) according to the manufacturer's protocol. Primers used in this study were purchased from Sunbiotech Co. Ltd. (Beijing, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in the comparative CT method to determine the relative changes in the target samples. All primers were as follows (forward and reverse, respectively): SIRT1, 5'-GGACTCCAAGGCCACGGATA-3' and 5'-GTT CGA GGA TCT GTG CCA ATC A-3'; CD40, 5'-CAC TGT ACG AGT GAG GCC TGT GA-3' and 5'-TTG CAC AAC CAG GTC TTT GGT C-3'; GAP DH, 5'-GCA CCG TCA AGG CTG AGA AC-3' and 5'-TGG TGA AGA CGC CAG TGG A-3'. The 2^{- $\Delta\Delta$ CT} method was used to calculate the quantitation of relative gene expression as described by the manufacturer.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. Data were subjected to statistical analysis by one-way analysis of variance (ANOVA), followed by LSD test. $P < 0.05$ was considered statistically significant.

Results

TNF- α reduced SIRT1 expression in CRL-1730 endothelial cells

To investigate the effect of TNF- α on SIRT1 expression, endothelial cells were treated with TNF- α (0, 10, 20, 40 μ g/L) for 8 h, and then the protein and mRNA expressions of SIRT1

Fig. 1. TNF- α reduced SIRT1 expression in CRL-1730 endothelial cells. (A) Concentration-dependent decrease of SIRT1 protein expression, and (B) concentration-dependent decrease of SIRT1 mRNA expression. The cells were treated with different concentrations of TNF- α for 8h. Then, the protein and mRNA expressions of SIRT1 were determined by western blot analysis and quantitative real-time PCR respectively. (C) Time-dependent decrease of SIRT1 protein expression, and (D) time-dependent decrease of SIRT1 mRNA expression. The cells were treated with TNF- α (10 μ g/L) for the indicated time. Then, the protein and mRNA expressions of SIRT1 were determined by western blot analysis and quantitative real-time PCR respectively. The values were presented as mean $\pm$ S.E.M. (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control (0 μ g/L TNF- α) group.

were determined by western blot analysis and quantitative real-time PCR respectively. As shown in Fig. 1A and B, TNF- α from 10 to 40 μ g/L concentration-dependently decreased SIRT1 expression at mRNA and protein levels in CRL-1730 endothelial cells, while unstimulated endothelial cells, referred to as control group in this study, showed a relatively high level in SIRT1 expression.

Based on the above results, a time-response experiment on SIRT1 expression in TNF- α -treated endothelial cells was performed. Endothelial cells were treated with TNF- α (10 μ g/L) for 0, 4, 8, 12, 24 h respectively. As shown in Fig. 1C and D, TNF- α at 10 μ g/L time-dependently decreased SIRT1 expression at mRNA and protein levels in CRL-1730 endothelial cells. SIRT1 expression was obviously down-regulated at 8 h, and reached the minimum at 12 h.

TNF- α didn't alter the subcellular localization of SIRT1 in CRL-1730 endothelial cells

Our preliminary studies has showed a predominant nuclear localization of SIRT1 in unstimulated CRL-1730 endothelial cells (Data not shown), but it's still unknown about the subcellular localization of SIRT1 in endothelial cells treated with TNF- α . To investigate the effect of TNF- α on subcellular localization of SIRT1 in CRL-1730 endothelial cells, endothelial cells were treated with TNF- α (10, 20 μ g/L) for 8h, and then immunofluorescence analysis was performed. As shown in Fig. 2, immunofluorescent labelling with a specific antibody demonstrated a predominant nuclear localization of SIRT1 in CRL-1730 endothelial cells treated with TNF- α (10, 20 μ g/L) for 8h. Of note, TNF- α at different concentrations did not alter the nuclear localization of SIRT1.

TNF- α induced CD40 expression in CRL-1730 endothelial cells

To investigate the effect of TNF- α on CD40 expression, endothelial cells were treated with TNF- α (0, 10, 20, 40 μ g/L) for 8 h, and then the protein and mRNA expressions of CD40

Fig. 2. TNF- α didn't alter the subcellular localization of SIRT1 in CRL-1730 endothelial cells. The cells were treated with TNF- α (10, 20 μ g/L) for 8h, and then immunofluorescence analysis was performed. SIRT1 was stained with anti-SIRT1 antibody. The nuclear protein was stained with DAPI. Merge represented the combined image of SIRT1 fluorescence and nucleus staining. Images were generated on a fluorescence microscope.

Fig. 3. TNF- α induced CD40 expression in CRL-1730 endothelial cells. (A) Concentration-dependent increase of CD40 protein expression, and (B) concentration-dependent increase of CD40 mRNA expression. The cells were treated with different concentrations of TNF- α for 8h. Then, the protein and mRNA expressions of CD40 were determined by western blot analysis and quantitative real-time PCR respectively. (C) Time-dependent increase of CD40 protein expression, and (D) time-dependent increase of CD40 mRNA expression. The cells were treated with TNF- α (10 μ g/L) for the indicated time. Then, the protein and mRNA expressions of CD40 were determined by western blot analysis and quantitative real-time PCR respectively. The values were presented as mean $\pm$ S.E.M. (n = 4). * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control (0 μ g/L TNF- α) group.

were determined by western blot analysis and quantitative real-time PCR respectively. As shown in Fig. 3A and B, TNF- α from 10 to 40 μ g/L concentration-dependently increased CD40 expression at mRNA and protein levels in CRL-1730 endothelial cells, while unstimulated endothelial cells, referred to as control group in this study, showed a low level in CD40 expression.

Based on the above results, a time-response experiment on CD40 expression in TNF- α -treated endothelial cells was performed. Endothelial cells were treated with TNF- α (10 μ g/L)

Fig. 4. Pharmacological modulation of SIRT1 regulated TNF- α -induced CD40 expression in CRL-1730 endothelial cells. (A and B) The cells were pretreated with SIRT1 activator RSV (20 μ M), and subsequently stimulated with TNF- α (10 μ g/L) for 8 h. (C and D) The cells were pretreated with class III HDACs inhibitor NAM (15 mM) or SIRT1 inhibitor Sirtinol (10 μ M), and subsequently stimulated with TNF- α (10 μ g/L) for 8 h. Then the protein expressions of SIRT1 and CD40 were determined by western blot analysis. The values were presented as mean $\pm$ S.E.M. (n = 3). * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control group. # p < 0.05, ## p < 0.01, ### p < 0.001 compared with TNF- α group.

for 0, 4, 8, 12, 24 h respectively. As shown in Fig. 3C and D, TNF- α at 10 μ g/L significantly time-dependently increased the protein expression of CD40 in CRL-1730 endothelial cells. CD40 expression was up-regulated at 8 h, and reached the maximum at 12 h.

Pharmacological modulation of SIRT1 regulated TNF- α -induced CD40 expression in CRL-1730 endothelial cells

To assess the association between SIRT1 and TNF- α -induced CD40 expression, endothelial cells were pretreated with SIRT1 activator RSV (20 μ M), class β HDACs inhibitor NAM (15 mM) or SIRT1 inhibitor Sirtinol (10 μ M), and subsequently stimulated with TNF- α (10 μ g/L) for 8 h. The protein expressions of SIRT1 and CD40 were determined by western blot analysis. As shown in Fig. 4A and B, pretreatment of endothelial cells with RSV increased SIRT1 expression, but attenuated CD40 expression induced by TNF- α in CRL-1730 endothelial cells. At the same time, pretreatment of endothelial cells with NAM and sirtinol decreased SIRT1 expression, but significantly augmented TNF- α -induced CD40 accumulation in CRL-1730 endothelial cells (Fig. 4C and D). Our results suggested that SIRT1 might regulate TNF- α -induced CD40 expression in CRL-1730 endothelial cells.

Genetic modulation of SIRT1 regulated TNF- α -induced CD40 expression in CRL-1730 endothelial cells

After endothelial cells were transiently transfected with SIRT1 siRNA or NC siRNA for 48 h, the protein expression of SIRT1 were obviously down-regulated by SIRT1 siRNA but not NC siRNA (Fig. 5A).

To prove the association between SIRT1 and TNF- α -induced CD40 expression, endothelial cells were stimulated with TNF- α (10 μ g/L) for 8 h after transfected with SIRT1 siRNA for 48 h, and then the protein expressions of SIRT1 and CD40 were determined by western blot analysis. As shown in Fig. 5B, SIRT1 expression were further down-regulated by

Fig. 5. Genetic modulation of SIRT1 regulated TNF- α -induced CD40 expression in CRL-1730 endothelial cells. (A) SIRT1 expression was obviously down-regulated by SIRT1 siRNA but not NC siRNA. The cells were transiently transfected with SIRT1 siRNA or NC siRNA for 48 h, and then the protein expression of SIRT1 was determined by western blot analysis. (B) TNF- α -induced increase of CD40 expression was significantly augmented by SIRT1 siRNA. The cells were stimulated with TNF- α (10 μ g/L) for 8 h after transfecting with SIRT1 siRNA for 48 h, and then the protein expressions of SIRT1 and CD40 were determined by western blot analysis. The values were presented as mean $\pm$ S.E.M. (n = 3). ** p < 0.01, *** p < 0.001 compared with control group. ## p < 0.01, ### p < 0.001 compared with TNF- α group.

SIRT1 siRNA compared with TNF- α group, while TNF- α -induced increase in CD40 expression was augmented by SIRT1 siRNA, these results suggested that SIRT1 inhibited TNF- α -induced CD40 expression in CRL-1730 endothelial cells.

SIRT1 regulated TNF- α -induced CD40 expression via NF- κ B pathway in CRL-1730 endothelial cells

Endothelial cells were pretreated with NF- κ B inhibitor PDTC (20 μ M), and subsequently stimulated with TNF- α (10 μ g/L) for 8 h, then the protein expression of CD40 were determined by western blot analysis. As shown in Fig. 6A, PDTC pretreatment attenuated TNF- α -induced CD40 expression in endothelial cells, which demonstrated that NF- κ B contributed to TNF- α -induced CD40 expression in CRL-1730 endothelial cells.

To discern the relationship of SIRT1, NF- κ B and CD40 accumulation induced by TNF- α , endothelial cells were stimulated with TNF- α (10 μ g/L) for 8 h after transfected with SIRT1 siRNA for 48 h, and then the protein expression of acetylated-NF- κ B p65 (Lys310) were determined by western blot analysis. As shown in Fig. 6B, TNF- α caused an increase in acetylated-NF- κ B p65 (Lys310) protein expression in endothelial cells, and which was abolished by SIRT1 siRNA. These demonstrated that SIRT1 inhibited TNF- α -induced acetylated-NF- κ B p65 (Lys310) protein expression. Collectively, these results suggested that SIRT1 might inhibit TNF- α -induced CD40 expression by deacetylating the RelA/p65 subunit of NF- κ B at lysine 310 in CRL-1730 endothelial cells.

Discussion

Emerging evidence has suggested that SIRT1 is highly expressed in vasculature, and SIRT1 activation improves endothelial function and suppresses vascular inflammation [21]. In the present study, unstimulated CRL-1730 endothelial cells showed a relatively high SIRT1

Fig. 6. SIRT1 regulated TNF- α -induced CD40 expression via NF- κ B pathway in CRL-1730 endothelial cells. (A) NF- κ B pathway mediated TNF- α -induced CD40 expression in CRL-1730 endothelial cells. The cells were pretreated with NF- κ B inhibitor PDTC (20 μ M), and subsequently stimulated with TNF- α (10 μ g/L) for 8 h. Then the protein expression of CD40 was determined by western blot analysis. (B) SIRT1 inhibited TNF- α -induced acetylated-NF- κ B p65 (Lys310) protein expression. The cells were stimulated with TNF- α (10 μ g/L) for 8 h after transfected with SIRT1 siRNA for 48 h, and then the protein expression of acetylated-NF- κ B p65 (Lys310) was determined by western blot analysis. The values were presented as mean $\pm$ S.E.M. (n = 3). **p < 0.01 compared with control group. ##p < 0.01 compared with TNF- α group.

Fig. 7. Schematic diagram of the regulation of SIRT1 on TNF- α induced-CD40 expression in CRL-1730 endothelial cells. TNF- α induced CD40 expression in CRL-1730 endothelial cells; NF- κ B pathway mediated TNF- α induced CD40 expression in CRL-1730 endothelial cells; TNF- α reduced SIRT1 expression in CRL-1730 endothelial cells; SIRT1 regulated TNF- α -induced CD40 expression in CRL-1730 endothelial cells by deacetylating the RelA/p65 subunit of NF- κ B at lysine 310.

expression, but treatment of CRL-1730 endothelial cells with TNF- α significantly decreased SIRT1 expression. TNF- α , as an inflammatory mediator, contributes to the development of atherosclerosis and the subsequent cardiovascular events by inducing inflammatory responses [22]. Our results showed that TNF- α didn't alter the nuclear localization of SIRT1 in CRL-1730 endothelial cells, but significantly decreased SIRT1 expression at mRNA and protein levels in time- and concentration-dependent manners in CRL-1730 endothelial cells. Shen et al. have revealed that treatment with LPS caused a significant decrease in SIRT1 expression, transcription, translation and activation in murine macrophages [23], and Arunachalam et al. also found that SIRT1 level and activity were both decreased after exposed to cigarette smoke extract and H₂O₂ in HUVECs [24]. What's more, reduced SIRT1 level has been found in human atherosclerotic vessels [25, 26], and in atherosclerotic animal models [7, 8]. The above studies suggest that the decrease in SIRT1 expression is involved in atherosclerotic processes *in vivo* and *in vitro*.

Our previous studies have showed that CD40 participates in inflammatory events of atherosclerosis *in vivo* and *in vitro*, and CD40 expression is increased in HUVECs treated with

CRP [27], and hydrogen peroxide [16]. In the present study, treatment with TNF- α significantly increased CD40 expression at mRNA and protein levels in time- and concentration-dependent manners in CRL-1730 endothelial cells. Our previous study has also reported that CD40 expression is obviously increased by TNF- α treatment in primary cerebral vascular endothelial cells and contributes to many neurological diseases [28]. So the finding of the present study supports the notion that CD40 participates in inflammatory processes of atherosclerosis. Resveratrol, a polyphenol in red wine, has been reported to have the ability to activate SIRT1. Other studies have revealed that resveratrol could inhibit IL-8 protein production induced by phorbol ester in human monocytic cells [29], and significantly decrease endothelin-1 and E-selectin mRNA expressions in high glucose cultured primary HUVECs [30]. In the present study, we also found that pretreatment with resveratrol attenuated CD40 expression induced by TNF- α in CRL-1730 endothelial cells. These results suggest that SIRT1 activation by resveratrol might inhibit the expressions of proinflammatory cytokines including CD40. What's more, Zhu et al. have shown that resveratrol reduced TNF- α -induced inflammation in fibroblasts, which is notably Sirt1 dependent [31]. Similarly, Csiszar et al. showed that resveratrol and SIRT1 over-expression attenuated the upregulation of inflammatory markers (ICAM-1, IL-6, IL-1 and TNF- α) induced by smoking, however the anti-inflammatory effects of resveratrol were significantly attenuated by knockdown of SIRT1 [32]. Recent studies have indicated that resveratrol activates SIRT1 without directly targeting it, so in order to further validate the above findings, we also pretreated endothelial cells with NAM (class β HDACs inhibitor) or sirtinol (SIRT1 inhibitor), and the results showed that NAM and sirtinol markedly increased the expression of CD40 induced by TNF- α in CRL-1730 endothelial cells. So these results further indicated the relationship between SIRT1 and CD40 in CRL-1730 endothelial cells. Moreover, liver-specific SIRT1 knockout mice develop hepatic inflammation when challenged with a high-fat diet [33], and addition of sirtinol or knocking down SIRT1 augmented proinflammatory cytokine release in cultured macrophage cell lines [23]. In the present study, we also found that knocking down SIRT1 by SIRT1 siRNA significantly augmented TNF- α -induced CD40 expression in CRL-1730 endothelial cells, and it proved that SIRT1 inhibited TNF- α -induced CD40 expression which was involved in inflammation-associated diseases including atherosclerosis. Besides suppressing inflammatory events, emerging evidence has also suggested that SIRT1 shows its anti-atherosclerosis effect by regulating blood lipids metabolism [34], improving endothelium relaxation function [7, 35, 36], reducing macrophage foam cell formation [7, 35-37], inhibiting vascular smooth muscle cell hypertrophy [38], and inhibiting arterial thrombosis and tissue factor expression (the key trigger of coagulation) in stimulated cells [39].

It's well known that the physiological functions of SIRT1 are mediated by deacetylation of a wide range of target proteins and some important transcription factors such as eNOS, NF- κ B, P53, FOXO transcription factors and peroxisome proliferator-activated receptor- γ [35, 40-44], and the anti-inflammatory properties of SIRT1 are closely related to its negative regulation of NF- κ B, a master transcription factor involved in expressions of proinflammatory cytokines [45]. The full transcriptional function of NF- κ B RelA/p65 requires acetylation at Lys310 [46]. However, SIRT1 physically interacts with the RelA/p65 subunit of NF- κ B by deacetylating RelA/p65 at lysine 310, and subsequently inhibits NF- κ B transcriptional activity [44]. Earlier reports have demonstrated that SIRT1 blocks the upregulation of NF- κ B signaling induced by amyloid-beta in microglia [47]. In the present study, TNF- α caused an increase in acetylated-NF- κ B p65 (Lys310) protein expression, but this effect was augmented by SIRT1 siRNA. These data suggested that SIRT1 might deacetylate the RelA/p65 subunit of NF- κ B at lysine 310, and then inhibit NF- κ B transcriptional activity in CRL-1730 endothelial cells treated with TNF- α . Furthermore, through NF- κ B inhibition, resveratrol at nutritionally relevant concentrations inhibits TNF- α -induced inflammatory gene expression [48, 49]. SIRT1 activation by resveratrol inhibits NF- κ B transcriptional activity and subsequently suppresses MCP-1 expression in TNF- α -induced adipocytes [48, 49]. In the present study, we also showed that pretreatment with PDTC (NF- κ B inhibitor) remarkably antagonized TNF- α -induced CD40 expression in CRL-1730 endothelial cells. Our previous study has also shown

that CRP-induced CD40 expression is mediated by NF- κ B pathway in HUVECs [18]. So these data suggested that NF- κ B pathway mediated TNF- α induced CD40 expression in CRL-1730 endothelial cells. Moreover, CR inhibited NF- κ B activation and NF- κ B-driven inflammatory gene expression in aged rat arteries via a SIRT1-dependent pathway [32, 50]. Ablation of SIRT1 in macrophages renders NF- κ B hyper-acetylated resulting in increased transcriptional activation of proinflammatory target genes [32, 50]. SIRT1 also regulated cigarette smoke-induced proinflammatory mediators (TNF- α and IL-8) expressions via RelA/p65 NF- κ B in macrophages and in rat lungs [51]. Taken together, the above results suggested SIRT1 might deacetylate the RelA/p65 subunit of NF- κ B at lysine 310, and then suppress TNF- α -induced CD40 expression in CRL-1730 endothelial cells.

In conclusion, our data provided the direct evidence that SIRT1 can inhibit TNF- α -induced CD40 expression in CRL-1730 endothelial cells by deacetylating the RelA/p65 subunit of NF- κ B at lysine 310. These findings might be pivotal for understanding the potential role of SIRT1 in modulating inflammatory events in atherosclerosis. Further investigation *in vivo* is needed to validate our findings.

Conflict of interest

The authors declare that there are no conflicts of interest.

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