SIRT4 suppresses the PI3K/Akt/NF-κB signaling pathway and attenuates HUVEC injury induced by oxLDL

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Received July 10, 2018; Accepted March 27, 2019

DOI: 10.3892/mmr.2019.10161

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Key words: sirtuin 4, atherosclerosis, inflammatory cytokine, nuclear factor-κB

Abstract. Atherosclerosis is a chronic and progressive disease. Its morbidity and mortality rates have demonstrated an increase in recent years. The present study aimed to explore the role of sirtuin (Sirt) 4 in the development of atherosclerosis. Alterations in SIRT4 expression in response to oxidized low density lipoprotein (oxLDL) were quantified in human umbilical vein endothelial cells (HuVECs) using western blotting. Cell counting kit-8 and flow cytometry assays were used in order to explore the effects of SIRT4 on HuVEC proliferation and apoptosis. The effect of SIRT4 on the expression of inflammatory factors in HuVECs was analyzed using ELISA. The expression and phosphorylation of proteins in the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/nuclear factor (NF)-κB pathway were comparatively analyzed using western blotting. Nuclear translocation of p65 NF-κB was examined using immunofluorescence. The present study indicated that oxLDL treatment decreased the expression of SIRT4 in HuVECs in a dose- and time-dependent manner. SIRT4 overexpression promoted oxLDL-induced HuVEC proliferation and inhibited cell apoptosis. Furthermore, SIRT4 overexpression suppressed the PI3K/Akt/NF-κB pathway by inhibiting PI3K phosphorylation and phosphorylated (p)-Akt, p-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α and p-p65 NF-κB expression; blocking p65 NF-κB nuclear translocation and decreasing interleukin (IL)-1β, IL-6, and tumor necrosis factor α expression in oxLDL-induced HuVECs. In conclusion, SIRT4 overexpression enhanced HuVEC survival, suppressed the PI3K/Akt/NF-κB signaling pathway and inhibited the expression of inflammatory cytokines in oxLDL-induced HuVECs.

Introduction

Atherosclerosis is a major disease that has a severe effect on human health. Its morbidity and mortality rates have demonstrated an increase in recent years (1,2). Atherosclerosis develops due to impairment of molecular and cellular activities, leading to the disruption of vascular homeostasis (3). Currently, this disease is primarily considered as a form of chronic inflammation, which arises in response to lipid accumulation. Macrophages are effector cells which stimulate vascular inflammatory reactions throughout the pathological process (4). An increasing number of factors reportedly participate in the inflammatory response in atherosclerosis (5,6); however, the detailed mechanisms underlying this process remain to be elucidated. Thus, an improved understanding of the pathophysiology of atherosclerosis in addition to the development of effective treatment methods are of great importance.

Sirtuin (SIRT) 4 is a member of the sirtuin protein family and is abundant in the heart, brain, kidney, liver and skeletal muscles (7). The Sirtuin family of proteins, from SIRT1 to SIRT7, serve key roles in the prevention of atherosclerosis (8). SIRT1 and SIRT6 inhibit foam cell formation and promote foam cell egress to prevent atherogenesis (9,10). SIRT2 may regulate macrophage polarization in order to inhibit atherosclerotic plaque progression (11). SIRT4, initially reported as not showing nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity (12), is localized in the mitochondrial matrix (13). It uses NAD to adenosine diphosphate ribosylate glutamate dehydrogenase (GDH) and suppresses GDH activity, limiting the generation of adenosine triphosphate (14). A previous study suggested that SIRT4 is a major regulator of lipid metabolism (15). Under nutrient-replete conditions, SIRT4 acts to repress fatty acid oxidation and promote lipid anabolism (16). In a previous study, it was demonstrated that lipopolysaccharide (LPS) treatment significantly decreased the expression of SIRT4 at mRNA and protein levels in a dose-dependent manner (17). In colorectal cancer, SIRT4 suppressed the proliferation, migration and invasion of cancer...
cells through the inhibition of glutamine metabolism via upregulation of E-cadherin expression (18). SIRT4 overexpression protected against diabetic nephropathy by preventing glucose-induced podocyte apoptosis and production of reactive oxygen species (19). Our previous study indicated that SIRT4 may inhibit inflammatory responses in human umbilical vein endothelial cells (HUVECs) (20).

Oxidized low density lipoprotein (oxLDL) reportedly induces atherosclerosis by triggering endothelial cell damage, and promoting lipid accumulation and proinflammatory responses (21). Nuclear factor (NF)-κB, a multifunctional transcription regulator, serves an important role in the inflammatory pathways of atherosclerosis, where expression of NF-κB and its downstream genes can be regulated via the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, which serves a vital role in the processes of cell proliferation, apoptosis and inflammation (22,23). The aim of the present study was to investigate the role of SIRT4 in the development of atherosclerosis. The results indicated that oxLDL reduced the expression of SIRT4 in HUVECs. In addition, it was identified that overexpression of SIRT4 may reverse oxLDL-induced cell proliferation inhibition, rescue oxLDL-induced apoptosis and attenuate the expression of pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α induced by oxLDL, possibly via inhibition of the PI3K/Akt/NF-κB signaling pathway.

Materials and methods

Cell culture and treatment. HUVECs were obtained from Capsugel, Morristown, NJ, USA). The cells were cultured in Endothelial Growth Basal Medium (EBM-2; Lonza Group, Ltd., Basel, Switzerland) supplemented with growth factors according to the manufacturer's protocols. To analyze the changes in SIRT4 expression in response to oxLDL (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. To analyze the changes in SIRT4 expression in response to oxLDL (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at various concentrations, cells were treated with oxLDL for 24 h at concentrations of 0, 10, 50 and 100 µM at 37°C. In order to analyze changes in SIRT4 expression in response to oxLDL treatment following different time periods, cells were treated with 50 µM oxLDL for 0, 12, 24 and 48 h at 37°C. Following treatment, cells were harvested and assessed for changes in SIRT4 expression using western blotting.

Stable overexpression of SIRT4 and treatment. The complete open reading frame of SIRT4 was cloned into the pLVX-ires-ZsGreen1 (Clontech Laboratories, Inc., Mountain View, CA, USA) plasmid (OV-SIRT4), using the empty vector as negative control (-NC), pLVX-SIRT4 was generated by transiently transfecting 293T cells (Beijing Zhongyuan Ltd., Beijing, China). Lentiviral production, concentration and titration were performed as follows: 293T cells (70-80% confluence) were seeded in 6-well plates, and 1.5 µg pLVX-ires-ZsGreen1 was transfected using the Lipofectamine® 2000 (40 µl; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Then, 293T cells were cultured for 48 h at 37°C. Lentivirus particles were directly collected and concentrated from the cell culture medium at 48 h following transduction by multi-steps of ultracentrifugation (50,000 x g at 4°C for 2 h). The titration (transduction units, TU) and multiplicity of infection of concentrated lentivirus particles were determined in 293T cells grown in 96-well plates by serial dilutions. For infection purposes, 2x10^6 HUVECs were divided into 2 groups and subcultured in 6-well culture plates for 24 h prior to transduction. For infection, the cell culture medium was removed and cells were washed twice with phosphate-buffered saline (PBS). Next, 0.5 ml of lentiviral suspension (1x10^8 IU/ml, multiplicity of infection=100) containing 8 µg/ml Polybrene was added to the cells. Cells were then incubated at 37°C overnight. Vector suspension was then aspirated from the cells and the transduced cells were added to 2 ml flask of fresh growth medium. Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO2. The growth medium was replaced after 24 h. After allowing the cells to incubate for 72 h at 37°C, HUVECs cells were passaged twice per week with growth medium containing 5 µg/ml puromycin to select for cells expressing the transduced vector. Positively screened cell lines were sub-cloned three times using limiting dilution (diluent: EB-2) and cultured in growth medium containing puromycin for 1 month to generate stable cell lines. HUVECs were infected by pLVX-SIRT4 and treated with 50 µM oxLDL. Following incubation for 48 h at 37°C and 5% CO2, HUVECs overexpressing SIRT4 and NC cells were harvested for western blotting.

Western blotting. Following treatment or infection, cells were lysed using a cell lysis buffer containing protease inhibitors [Tris-HCl (pH 7.5) 50 mM, NaCl 250 mM, EDTA 10 mM, NP-40 0.5%, Leupeptin 10 µM, PMSF 1 mM and NaF 4 mM]. The protein concentration was measured using a bichinonic acid protein assay kit (Thermo Fisher Scientific, Inc.). Soluble lysate was mixed with loading buffer and boiled for 5 min. Equal amounts of the protein samples (50 ng/lane) were separated via 10% SDS-PAGE and transferred on to polyvinylidene difluoride membranes. Membranes were blocked with PBS, containing 10% non-fat dry milk, overnight at 4°C and incubated with anti-SIRT4 antibody (1:1,000; cat. no. ab124521), anti-GAPDH antibody (1:10,000; cat. no. 5174), anti-Pi3K antibody (1:500; cat. no. 4249), anti-Akt antibody (1:1,500; cat. no. 4691), anti-phosphorylated (p)-Akt antibody (1:500; cat. no. 4060), anti-p-inhibitor of κB (IκBα) antibody (1:500; cat. no. 2859), anti-p-p65 NF-κB antibody (1:500; cat. no. 8242) and anti-p-p65 NF-κB antibody (1:500; cat. no. 3033) for 2 h at 25°C. Anti-SIRT4 was obtained from Abcam (Cambridge, MA, USA); all other primary antibodies were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA). Membranes were washed with TBS-0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G heavy and light chain secondary antibodies (1:10,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature. This process was followed by the development of protein bands for visualization. Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to quantify relative protein densities. GAPDH was used as the loading control. Each experiment was replicated three times.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting Kit-8 assay (CCK-8; Beyotime Institute of
Biotechnology, Shanghai, China), according to the manufacturer's protocols. HUVECs stably overexpressing SIRT4 and control cells were cultured in 96-well plates and treated with 50 μM oxLDL for 48 h at 37°C and 5% CO₂. Then, 10 μl of CCK-8 reagent was added to each well and mixed gently; cells were then incubated at 37°C for 4 h. The absorbance was evaluated at 450 nm using a microplate reader. Survival rate=optical density (OD)450ox-SIR4 group/OD450nc group. Each experiment was repeated three times.

**Apoptosis assay.** Cell apoptosis was analyzed using flow cytometry. HUVECs stably overexpressing SIRT4 and NC cells were cultured and treated with 50 μM oxLDL for 48 h at 37°C and 5% CO₂. The cells were collected and the assay was performed using a Propidium Iodide (PI)/Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Nanjing Keygen Biotech Co., Ltd., Nanjing, China). Annexin V-FITC (5 μl) and PI (5 μl) were added, and cells were incubated at 25°C in the dark for 15 min. Within 1 h, the apoptotic cells was then assessed using a flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using FlowJo version 10.07 software (FlowJo LLC, Ashland, OR, USA). The apoptotic rate was defined as the percentage of cells in the upper and lower right quadrants. Each experiment was repeated three times.

**Immunofluorescence.** HUVECs stably overexpressing SIRT4 and NC cells (4x10⁵ cells/ml) were seeded into 24-well plates and treated with 50 μM oxLDL for 48 h at 37°C and 5% CO₂. Following treatment, the cells were washed with PBS and then fixed with 4% paraformaldehyde for 15 min at 37°C. The cell membranes were then permeabilized using 0.3% Triton X-100 in PBS with 0.2% (V/V) Tween-20 (PBST) on ice for 15 min, followed by blocking with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) and 2.5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in PBST and were then incubated for 2 h at 25°C with rabbit anti-NF-κB p65 antibody (1:100; cat. no. 8242; Cell Signaling Technology, Inc.). Next, cells were washed three times with PBST and incubated with the Alexa Fluor® 594-conjugated secondary antibody (1:200; cat. no. ab150084; Abcam) for 1 h at room temperature. Following three washes with PBST, the cells were then counterstained with DAPI (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 min at 25°C. The cells were then visualized and images captured via fluorescence microscopy (magnification, x400; Leica Microsystems GmbH, Wetzlar, Germany).

**Enzyme-linked immunosorbent assay (ELISA) for cytokine detection.** HUVECs stably overexpressing SIRT4 and NC cells (4x10⁵ cells/ml) were seeded into 24-well plates, followed by treatment with 50 μM oxLDL for 48 h at 37°C and 5% CO₂. Cell-free supernatants were collected and used in an assay for the detection of cytokines. The concentrations of the cytokines IL-1β (E-EL-H0149e), IL-6 (E-EL-H0102c) and TNF-α (E-EL-H0109c) were determined using commercially available ELISA kits (eBioscience; Thermo Fisher Scientific, Inc.). The entire procedure was performed according to the manufacturer's protocols. Each experiment was replicated three times.

**Statistical analysis.** All statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Results are presented as the mean ± standard deviation, and experiments were repeated in triplicate. Student's t-test was performed for comparison between two groups. Multiple groups were compared using one-way analysis of variance, followed by a least significant difference post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**OxLDL reduces the expression of SIRT4 in HUVECs.** In order to investigate changes in SIRT4 expression in response to oxLDL, HUVECs were treated with oxLDL at various concentrations. Western blotting results demonstrated that oxLDL treatment significantly reduced SIRT4 expression in HUVECs, compared with in control cells treated with 0 μM oxLDL. Higher oxLDL concentrations resulted in lower SIRT4 expression levels indicating a dose dependent association between oxLDL concentrations and SIRT4 expression (Fig. 1A). Next, HUVECs were treated with the same oxLDL concentration (50 μM) for different periods of time. Western blotting results demonstrated that oxLDL treatment significantly reduced SIRT4 expression in HUVECs compared with control cells treated with 0 μM oxLDL, in a time dependent manner; longer durations of treatment resulted in lower SIRT4 expression levels (Fig. 1B).

**Stably overexpressing SIRT4 in HUVECs.** Cells overexpressing SIRT4 were selected by screening and treated with 50 μM oxLDL for 48 h. SIRT4 expression was determined via western blotting. SIRT4 expression levels were significantly higher in cells overexpressing SIRT4, compared with the NC group following oxLDL treatment for 48 h (P<0.001; Fig. 2). The results indicated successful overexpression of SIRT4 in HUVECs.

**Overexpression of SIRT4 reverses oxLDL-induced inhibition of cell proliferation.** To investigate the effect of SIRT4 overexpression in response to oxLDL on HUVEC proliferation, HUVECs overexpressing SIRT4 and NC HUVECs were treated with 50 μM oxLDL for 48 h. Cell proliferation was assessed using a CCK-8 assay. The survival rate was significantly higher in HUVECs overexpressing SIRT4, compared with NC HUVECs following oxLDL treatment (Fig. 3A). The results indicated that SIRT4 overexpression may reverse oxLDL-induced inhibition of cell proliferation. In order to explore the effects of SIRT4 overexpression in response to oxLDL on HUVEC apoptosis, HUVECs overexpressing SIRT4 and NC HUVECs were treated with 50 μM oxLDL for 48 h. Cell apoptosis was examined via flow cytometry. Following oxLDL treatment, the apoptotic rate significantly decreased in HUVECs overexpressing SIRT4 compared with in NC HUVECs (Fig. 3B and C). These results indicated that overexpression of SIRT4 may inhibit oxLDL-induced apoptosis.

**Overexpression of SIRT4 inhibits the PI3K/Akt/NF-κB signaling pathway.** In order to elucidate the role of SIRT4 in regulating the PI3K/Akt/NF-κB signaling pathway,
HuVECs overexpressing SIRT4 and NC HuVECs were treated with 50 µM oxLDL for 48 h. Western blotting analysis was conducted to quantify protein expression associated with the PI3K/Akt/NF-κB signaling pathway. Compared with the NC, SIRT4 overexpression suppressed the PI3K/Akt/NF-κB pathway in HuVECs by inhibiting PI3K expression (Fig. 4A and B). It also significantly inhibited p-Akt, p-IκBα and p-P65 NF-κB expression compared with the control. P65 NF-κB nuclear translocation was analyzed via immunofluorescence. Immunofluorescence analysis revealed that P65 NF-κB was largely expressed in the nucleus, while a small quantity was expressed in the cytoplasm. These results suggest that OV-SIRT4 may inhibit P65 NF-κB nuclear translocation (Fig. 4C).

Overexpression of SIRT4 attenuates the expression of inflammatory cytokines induced by oxLDL. In order to further confirm the effect of SIRT4 overexpression on endothelial activation in response to inflammatory cytokines, HuVECs overexpressing SIR4 and NC HuVECs were treated with 50 µM oxLDL for 48 h. The expression of IL-1β, IL-6 and TNF-α were assessed using ELISA. Compared with NC HuVECs, the expression levels of IL-1β, IL-6 and TNF-α were significantly decreased in HuVECs overexpressing SIRT4 (Fig. 5). The results indicated that SIRT4 overexpression attenuated the expression levels of IL-1β, IL-6 and TNF-α induced at the protein level by oxLDL.

Discussion

Previous studies have indicated that endothelial dysfunction may serve a vital role during the initial stages of atherosclerosis (6,24). Although much progress has been made in studies of pathophysiology and clinical aspects of atherosclerosis, the detailed mechanism underlying this disease remains to be elucidated (25,26). OxLDL reportedly induces endothelial cell injury and dysfunction (27). In the present study, HuVECs were stimulated by oxLDL to mimic the conditions of damage and inflammation of endothelial cells. The results demonstrated that oxLDL may reduce SIRT4 expression in HuVECs in a dose- and time-dependent manner. SIRT4 is a member of the sirtuin protein family which is able to improve vascular smooth muscle and endothelial cell injury induced by lipid deposition, oxidative stress and inflammation, thus opposing...
Figure 3. Effect of SIRT4 on HUVEC proliferation and apoptosis following 50 µM oxLDL treatment. Each experiment was replicated three times. (A) Cell proliferation was analyzed using CCK8 reagent following 50 µM oxLDL treatment at 48 h in overexpressed SIRT4-HUVECs and NC-HUVECs. (B and C) Cell apoptosis was analyzed using flow cytometry following 50 µM oxLDL treatment at 48 h in overexpressed SIRT4-HUVECs and NC-HUVECs. Upper right and lower right quadrants contained the apoptotic cells (***P<0.001 vs. NC). CCK-8, Cell Counting Kit 8; FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells; nc, negative control; oxLDL, oxidized low density lipoprotein; PI, propidium iodide; SIRT4, sirtuin 4.

Figure 4. PI3K/Akt/NF-κB signaling pathway, activated by oxLDL, was suppressed by SIRT4 overexpression in HUVECs. (A and B) Expression of PI3K, p-Akt, t-Akt, p-IκBα, t-IκBα, p-p65 NF-κB, p65 NF-κB proteins were detected by western blotting following 50 µM oxLDL treatment at 48 h in overexpressed SIRT4-HUVECs and NC-HUVECs. Each experiment was replicated three times (***P<0.001 vs. NC). (C) P65 NF-κB nuclear translocation was examined via immunofluorescence in overexpressed SIRT4-HUVECs, NC-HUVECs and HUVECs (magnification, x400). HUVECs, human umbilical vein endothelial cells; IκBα, inhibitor of κBα; NF, nuclear factor; NF, nuclear factor, nc, negative control; oxLDL, oxidized low density lipoprotein; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; SIRT4, sirtuin 4; p-, phosphorylated; t-, total.
In conclusion, the present study provided notable evidence that SIRT4 overexpression attenuated the expression of IL-1β, IL-6 and TNF-α induced by oxLDL by suppressing the PI3K/Akt/NF-κB pathway, which improves the inflammatory environment induced by oxLDL and enhances the survival of HUVECs.

The present study involved several limitations. First, all experiments were performed using one cell line and, therefore, it is possible that the conclusions are specific to this cell line only and may not apply to atherosclerosis per se. Thus, further validation of these findings using more cell and animal models are required in subsequent studies. Additionally, the potential pathways that mediate the effects of oxLDL on SIRT4 and the effects of SIRT4 on the PI3K/Akt pathway remain to be elucidated. Whether SIRT4 attenuates oxLDL-induced HUVEC apoptosis via the PI3K/Akt/NF-κB signaling pathway requires further investigation.

In conclusion, the present study provided notable evidence suggesting that SIRT4 overexpression enhanced HUVEC survival, suppressed the PI3K/Akt/NF-κB signaling pathway and inhibited inflammatory cytokine expression induced by oxLDL. These results may serve as a theoretical basis to provide novel insight into the treatment of atherosclerosis.

Acknowledgements

Not applicable.

Funding

This study was supported by the grant from Jiangxi Province Science Foundation for Youths (grant no. 20161BAB215257) and the Science and Technology Program by the Health and Family Planning Commission of Jiangxi Province (grant no. 20162304).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YT, SY and HL conceived and designed the present study. MC, YW and JX developed the methodology. YT, SY, MC,
YW and JX completed the experiment and collected the data. YT, SY and Hl analyzed and interpreted the data. YT and YW and JX completed the experiment and collected the data.

**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.

**References**


