SIRT1 prevents atherosclerosis via liver-X-receptor and NF-κB signaling in a U937 cell model

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Abstract. Atherosclerosis is a chronic immunoinflammatory disease associated with blood lipid disorders. Previous studies in mice have demonstrated that liver X receptor (LXR)-ATP-binding cassette (ABC) A1/ABCG1/C-C chemokine receptor type 7 (CCR7) and nuclear factor κB (NF-κB) signaling pathways are important for atherosclerotic plaque formation. In addition, Sirtuin 1 (SIRT1) has been reported as a key regulator in the protection from risk of atherosclerosis. However, the exact mechanism by which SIRT1 prevents atherosclerosis remains largely unknown. To explore the possible mechanisms, the expression of SIRT1 and the association between SIRT1, LXR and NF-κB in the process of foam cell formation was investigated in an *in vitro* human mononuclear U937 cell line. Monocyte-derived foam cells were induced by palmitate and Ox-LDL treatment. Oil Red O staining revealed an accumulation of a large number of lipid droplets in foam cells. Results of reverse transcription polymerase chain reaction (RT-PCR) revealed that SIRT1 expression was downregulated during foam cell formation. In addition, the expression of LXRα and its targets, ABCA1, ABCG1 and CCR7, were downregulated. However, NF-κB and its targets, tumor necrosis factor α (TNFα) and interleukin (IL)-1β, were upregulated in foam cells. Following activation of SIRT1 by SRT1720, the expression of LXRα and its targets increased, whereas expression of NF-κB and its targets decreased. Furthermore, the formation of foam cells was blocked. The SIRT1 inhibitor, nicotinamide, was found to eliminate the effects of SRT1720. Results of the present study indicate that SIRT1 may prevent the formation and progression of atherosclerosis by enhancing the LXR-ABCA1/ABCG1/CCR7 and inhibiting the NF-κB pathways.

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Introduction

Atherosclerotic arterial disease is the leading cause of morbidity and mortality in Western countries and is rapidly increasing in developing nations (1). An important step in atherogenesis is the infiltration of monocytes within the subendothelial space of large arteries and phagocytosis of local lipids. This is followed by differentiation into monocyte-derived foam cells, which simultaneously release large amounts of inflammatory cytokines that further promote monocyte aggregation. These steps contribute to a vicious cycle and are known to directly result in the development of myocardial infarction and stroke (2). In this process, lipid metabolism disorders and the inflammatory response were considered key factors in atherosclerosis formation.

A number of previous studies have described a mouse model of regression (2-4). First, plaques were developed in ApoE null ($ApoE^{-/-}$) mice and a segment of thoracic aortic or aortic arch was transplanted into the abdominal aorta of a wild type (WT) recipient, rapidly altering the plasma lipid environment from hyperlipidemia to a normal lipid level. As a control, an aortic segment was transplanted into an $ApoE^{-/-}$ mouse. In the regression environment (WT recipient), the majority of foam cells disappeared from plaques after 3 days, with no longer visible after 1 month (5). By contrast, in the progression environment ($ApoE^{-/-}$ recipient), plaque size and foam cell content increased in a time-dependent manner.

A more recent study observed that C-C chemokine receptor type 7 (CCR7) is induced in foam cells and is functionally required for regression (6). In addition, in the regression environment, foam cells were found to overexpress liver X receptor (LXR) α and ATP-binding cassette (ABC) A1 and scavenger receptor BI(SR-BI). Inflammation-related factors, including nuclear factor κB (NF-κB) targets, vascular cell adhesion protein 1, monocyte chemotactic protein 1 and other inflammatory factors, were downregulated at the mRNA level *in vivo*.

NF-κB, is a core nuclear transcription factor in the inflammatory response, enhancing the expression of various cytokines and chemical factors in the formation of atherosclerosis and promoting initiation and progression of atherosclerotic

lesions (7). In a previous study, gene knockout of the NF-κB subunit, p50, was found to lead to a significant reduction in atherosclerotic lesions and almost no foam cells were observed in the lesions of a mouse atherosclerosis model ($LDLR^{-/-}$) (8).

Caloric restriction (CR) is known to slow the aging process and may have the potential to prevent a wide range of diseases associated with aging, including to reduce the risk of atherosclerotic disease (9,10). One of the key factors involved in the mechanism of CR is Sirtuin 1 (SIRT1) (11). SIRT1 transgenic mice exhibit a number of phenotypes similar to mice on a CR diet. Mice are leaner than littermate controls, more metabolically active, exhibit reductions in blood cholesterol, adipokines, insulin and fasting glucose levels and have increased glucose tolerance (12). SIRT1 activation represents a promising therapeutic approach for the prevention of atherosclerosis via multiple pathways (13,14).

Li *et al* previously reported that SIRT1 positively regulates LXRs by deacetylation at lysine K432 and is important for cholesterol homeostasis. $SIRT1^{-/-}$ cells were found to exhibit defective cholesterol efflux and reduced ABCA1 gene expression (15). In addition, a more recent study found that SIRT1 overexpression in transgenic mice inhibited NF-κB activity induced by high-fat foods and reduced specific proinflammatory cytokines, including tumor necrosis factor α (TNFα) and interleukin (IL)-6, promoting fatty liver (16). By contrast, SIRT1-knockout mice administered with a high-fat diet were found to be prone to inflammation of the liver and fatty liver formation (17).

Therefore, in the present study, we hypothesized that SIRT1 expression is inhibited during monocyte-derived foam cell formation, resulting in downregulation of LXR-ABCA1/ABCG1/CCR7 and upregulation of NF-κB proinflammatory signaling pathways, leading to foam cell formation and blockage of foam cell elimination from atherosclerotic plaques. To verify this, mRNA and protein levels of genes involved in monocyte-derived foam cell formation in U937 cells treated with palmitate and Ox-LDL were analyzed and observations confirmed the association of SIRT1 with LXR and NF-κB.

Materials and methods

Materials. RPMI-1640 medium was obtained from Gibco-BRL (Carlsbad, CA, USA). Oil Red O was purchased from Bio Basic Inc. (Markham, ON, Canada). Sodium palmitate, nicotinamide and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SRT1720 was purchased from Selleck Chemicals LLC (Houston, TX, USA). For cell treatment, SRT1720 stock solution was prepared in ultra-pure water; stock solution of sodium palmitate was dissolved in a bovine serum albumin/Hanks Balanced Salt Solution (BSA/HBSS) mixture, as described previously (18,19).

Cell culture and treatment. Human mononuclear U937 cells were grown and maintained in culture in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) in a 5% CO_2 atmosphere at 37˚C. For each experiment, U937 cells were plated in 6-well plates ($3x10^5$ cells/ml) in RPMI-1640 medium containing 0.2 μM/l PMA. After 24 h, cells were harvested.

Cells were divided into five groups: the control, CR, high fat (HF) + OxLDL, HF + Ox-LDL + SRT1720 and HF + Ox-LDL + SRT1720 (SRT) + nicotinamide (Nam) groups. Control cells were cultured in RPMI-1640 medium containing 0.45% glucose only. In HF groups, cells were exposed to a medium containing 0.2 mM palmitate and 0.45% glucose. Similarly, for groups treated with Ox-LDL, cells were exposed to medium containing 80 μg/ml Ox-LDL. CR groups were treated with medium containing 0.1% glucose. For groups without palmitate, the same concentration of BSA/HBSS mixture was added to the medium. In all the experiments, cells were incubated for 24 h.

Cell viability assay. The viability of U937 cells was measured by an MTT assay. The cells were grown in 96-well plates ($2.5x10^4$ cells/well) with 100 μl medium. The medium was refreshed with various concentrations of sodium palmitate (0.05-0.8 mM). For the control group, the same concentration of vehicle was added to the medium. Following culture for 24 h, cells were incubated with MTT for 4 h at 37˚C. Next, triple liquid (10% SDS, 5% isobutanol, 0.012 mol/l hydrochloric acid, dissolved in distilled water) was added to each well. The absorbance of samples was measured at 570 nm using a microtiter plate reader (Multiskan MK3, Thermo Scientific, Helsinki, Finland). All the experiments were performed independently in triplicate.

Oil Red O staining. Cells were fixed in 4% paraformaldehyde in PBS for 20 min and stained with freshly diluted Oil Red O solution (6 parts 0.1% Oil Red O in isopropyl alcohol and 4 parts water) for 2 h at room temperature. Cell images were captured under a microscope. For quantitative analysis of cellular triglycerides, $2.5x10^4$ cells were stained with freshly diluted Oil Red O solution for 2 h at room temperature in every Eppendorf tube. Oil Red O was removed by centrifugation and cells were extensively rinsed with water. Excess water was evaporated by placing the stained culture in an incubator at 37˚C. Next, 200 μl isopropyl alcohol was added to every EP tube. The extracted dye was immediately removed by gentle pipetting and absorbance was monitored using a spectrophotometer at 510 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total cellular RNA was isolated from cells using an RNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using a cDNA synthesis kit (TransGen Biotech Co., Ltd., Beijing, China). Primer sequences are presented in Table I and were synthesized by GeneCore Biotechnologies Co., Ltd. (Shanghai, China). Each PCR was performed with 1 μl cDNA product and 20 pM of each primer in a final volume of 25 μl using a PCR kit (Aidlab Biotechnologies Co., Ltd.) as follows: initial denaturation at 94˚C for 3 min; 32 or 35 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 58˚C and elongation for 1 min at 72˚C; followed by a final extension step for 5 min at 72˚C. PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining. The relative intensi-

Table I. Specific primers used in PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
SIRT1	AGTGGCATTCCAGACTTCAGA	GGGCTTGTAGTTTCCAGGGTA
NF-κB	ACATGGTGGTCGGCTTCGCA	TGCAGAGCTGCTTGGCGGAT
IL-1β	GGACAGGATATGGAGCAACAAG	TTCAACACGCAGGACAGGTA
TNFα	TCAGCAAGGACAGCAGAGG	CCACGATCAGGAAGGAGAAGA
LXRα	TCTGCGGTGGAGCTGTGGAA	TGACGCTGGGCGGAAGAAT
ABCA1	GGAGCAGGCAATCATCAG	ACACGGACAGGAAGACAA
ABCG1	GGTCATCCTCTCCATCTATG	CAATCTGCCTACATCTTCCT
CCR7	ACACCAGACAGACAACAC	CTCACCAAGCCAAGAAGT
18S rRNA	TTGGTGGAGCGATTTGTCTG	AATGGGGTTCAACGGGTTAC

PCR, polymerase chain reaction; SIRT1, Sirtuin 1; NF-κB, nuclear factor κB; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α; LXRα, liver X receptor α; ABC, ATP-binding cassette; CCR7, C-C chemokine receptor type 7.

ties of the bands were quantified by densitometric analysis and normalized against corresponding 18S band densities.

Statistical analysis. All experiments were performed at least three times. Values are expressed as mean ± SD. Results were analyzed with unpaired Student's t-test or one-way ANOVA using the statistical software GraphPad Prism 5.01. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of palmitate on cell viability and cellular triglyceride accumulation. The effect of palmitate on cell viability was determined by an MTT assay (Fig. 1A) and 0.05 mM palmitate was revealed to slightly promote cell growth. Cell survival rate decreased with increasing concentrations of palmitate, for example, 0.2 and 0.4 mM palmitate decreased cell viability in a dose-dependent manner by 15.75±3.07 and 66.97±8.53%, respectively. Quantitative analysis of cellular triglycerides was performed to investigate the dose-dependent effect of palmitate on lipid accumulation in U937 cells. As demonstrated in Fig. 1B, ≥0.2 mM palmitate was found to significantly increase cellular lipid accumulation compared with the control. Oil Red O staining revealed that an increased number and larger lipid droplets accumulated in cells cultured with 0.2 mM palmitate (Fig. 1C). Based on these observations, 0.2 mM palmitate was used to induce the cell model of monocyte-derived foam cell formation to achieve maximal fat accumulation with minimal cytotoxicity.

mRNA expression of SIRT1 and its target genes during foam cell formation. The foam cell model was induced by palmitate and Ox-LDL treatment (20,21). U937 cells were cultured in control, CR or HF + Ox-LDL for 24 h and mRNA expression of SIRT1, LXRα, NF-κB and their targets was assessed by RT-PCR. As demonstrated in Fig. 2, the expression of SIRT1, LXRα and its targets, ABCA1, ABCG1 and CCR7, was downregulated; however, NF-κB and its targets, IL-1β and TNFα, were upregulated in the HF + OxLDL culture. By contrast, in the CR group, SIRT1 expression was upregulated, whereas NF-κB and its targets, IL-1β and TNFα, were downregulated. Oil red O staining revealed that triglyceride accumulation in cells of the HF + OxLDL group was significantly higher than that of the control and CR groups (Fig. 3).

mRNA expression of LXRα, NF-κB and their targets following activation of SIRT1 under HF + Ox-LDL culture conditions. Results of RT-PCR demonstrated that the expression of SIRT1, LXRα and its targets, ABCA1, ABCG1 and CCR7, was downregulated; however, NF-κB and its targets, IL-1β and TNFα, were upregulated 24 h after incubation in the HF culture with 80 μg/ml Ox-LDL. SRT1720 has been identified as a specific activator of SIRT1 and the activity of SRT1720 is increased 1,000-fold compared with that of resveratrol (22). Following stimulation with 6.0 μM/ml SRT1720 for 24 h, the expression of SIRT1 was unchanged. However, the expression of LXRα and its target genes (ABCA1, ABCG1 and CCR7)increased, whereas expression of NF-κB and its target genes (IL-1β and TNFα) decreased. Treatment for 24 h with 20 mM nicotinamide, the most potent inhibitor of Sir2 enzymes (23), was found to eliminate the effects of SRT1720 (Fig. 4).

Discussion

An increasing number of studies have demonstrated that SIRT1 is a key regulator in protection from atherosclerosis formation and progression (13,14). SIRT1 not only regulates inflammatory processes and cholesterol metabolism in macrophages, but also suppresses the expression of the scavenger receptor Lox-1 in macrophages, reduces the uptake of Ox-LDL and prevents monocyte-derived foam cell formation (13,24). However, the mechanisms by which these processes are mediated remain unknown. In the current study, the mechanisms of SIRT1 in the prevention of atherosclerosis were analyzed.

The function of peripheral blood monocyte infiltration into the subendothelial space is to clear local lipids. During atherosclerosis, these cells not only lose their clearance function, but are also unable to be removed from atherosclerotic plaques to

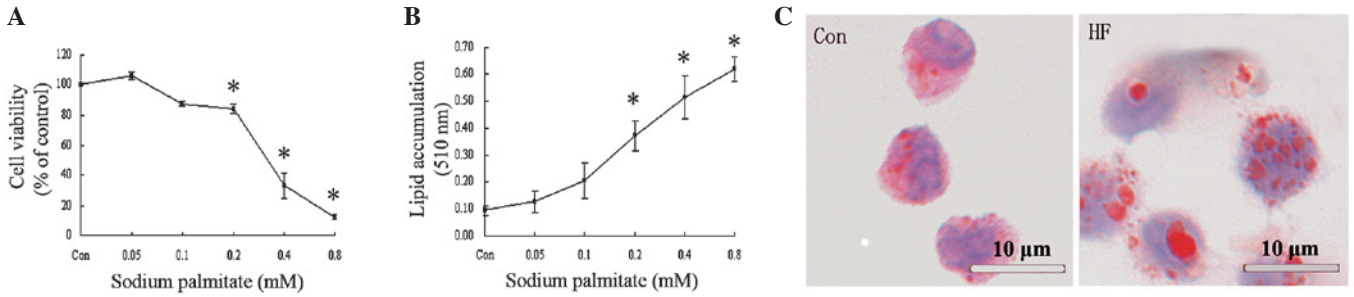

Figure 1. Effects of palmitate on cell viability and cellular triglyceride accumulation. (A) Dose-dependent effects of palmitate on cell survival. U937 cells were exposed to various concentrations of palmitate for 24 h and cell viability was determined by MTT analysis. Data are presented as the mean ± SD of three independent experiments run in triplicate. (B) Quantitative analysis of fat accumulation. Data are presented the as mean ± SD of three independent experiments.*P<0.05 vs. control. (C) Oil Red O staining of U937 cells in Con and 0.2 mM palmitate culture (HF). Con, control; HF, high fat.

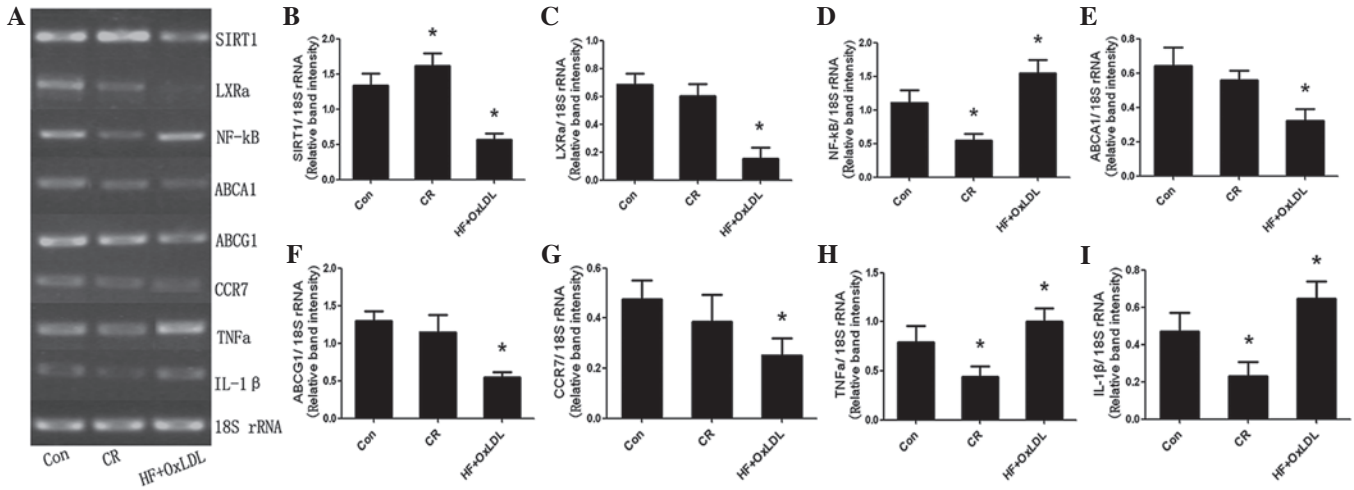

Figure 2. mRNA expression levels in control, CR and HF + OxLDL cultures, as determined by RT-PCR. (A) Electrophoresis and quantification of (B) SIRT1, (C) LXRα, (D) NF-κB, (E) ABCA1, (F) ABCG1, (G) CCR7, (H) TNFα and (I) IL-1β expression. *P<0.05 vs. control. Con, control; CR, caloric restriction; SIRT1, Sirtuin 1; NF-κB, nuclear factor κB; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α; LXRα, liver X receptor α; ABC, ATP-binding cassette; CCR7, C-C chemokine receptor type 7; HF, high fat.

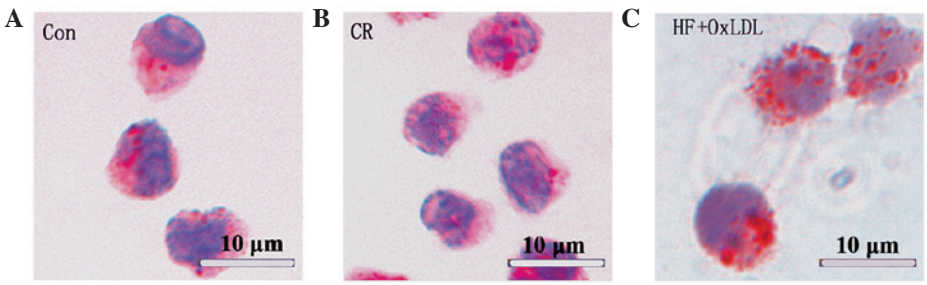

Figure 3. Oil Red O staining of U937 cells in (A) control, (B) CR and (C) HF+OxLDL cultures. Con, control; CR, caloric restriction; HF, high fat.

return to the lymphatic system or bloodstream. Regardless of this, monocytes within the arterial wall may be able to maintain their original clearance potential, although it may be blocked or inhibited. de Kreutzenberg *et al* (20) found that mRNA and protein expression of SIRT1 in peripheral blood mononuclear cells was significantly reduced in association with insulin resistance and metabolic syndrome. Song *et al* (25) also revealed that the expression of SIRT1 in blood mononuclear cells was significantly lower in patients with high cholesterol, hyperglycemia or diabetes than in healthy individuals. By contrast, under CR conditions, SIRT1 expression was increased (26). In the present study, expression of SIRT1 was found to decrease in high glucose and high lipid environments, representative of the blood of patients with atherosclerosis, compared with cells cultured in control conditions. In addition, SIRT1 expression significantly increased under conditions of CR. These results are consistent with previous studies and demonstrate that SIRT1 downregulation may be associated with development of atherosclerosis.

LXR serves as a cholesterol sensor to protect the organism from cholesterol overload. LXR is deacetyled by SIRT1 at a single conserved lysine (K432 in LXRα and K433 in LXRβ)

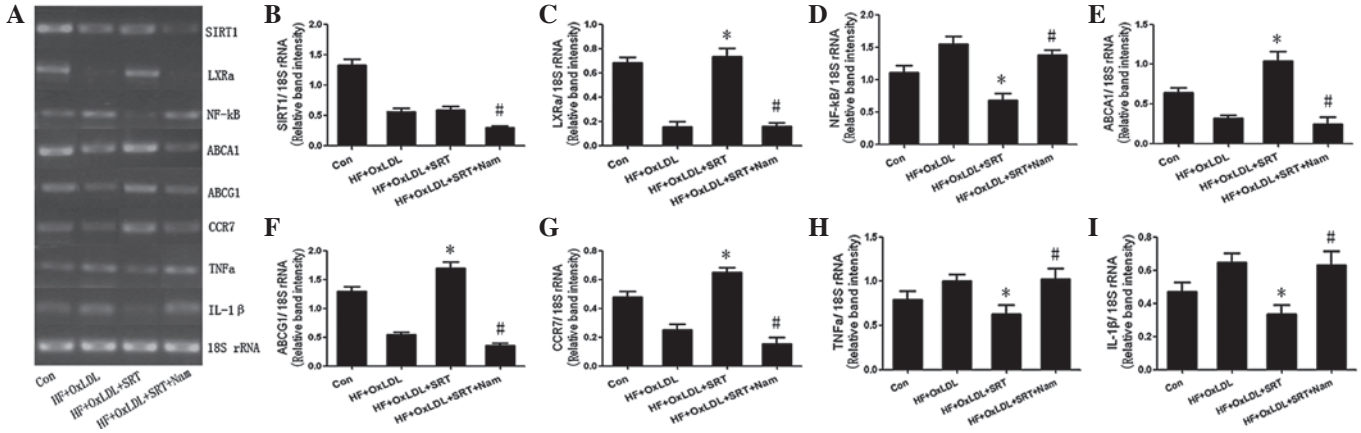

Figure 4. mRNA expression levels in control, HF + OxLDL, HF + OxLDL + SRT and HF + OxLDL + SRT + Nam cultures, as determined by RT-PCR. (A) Electrophoresis and quantification of (B) SIRT1, (C) LXRα, (D) NF-κB, (E) ABCA1, (F) ABCG1, (G) CCR7, (H) TNFα and (I) IL-1β expression. *P<0.05, vs. HF + OxLDL; #P<0.05, vs. HF + OxLDL + SRT. SIRT1, Sirtuin 1; NF-κB, nuclear factor κB; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α; LXRα, liver X receptor α; ABC, ATP-binding cassette; CCR7, C-C chemokine receptor type 7; HF, high fat; CR, caloric restriction; Con, control; SRT, SRT1720; Nam, nicotinamide.

adjacent to the ligand-regulated activation domain. SIRT1 interacts with LXR and promotes its deacetylation and subsequent activation (17). In the present study, expression of LXRα was found to be significantly reduced, consistent with SIRT1 expression, in U937 cells during foam cell formation. In addition, exposure of cells to 6.0 μM/ml SRT1720 was observed to lead to rapid activation of SIRT1, which subsequently enhanced expression of LXRα and its target genes, ABCA1, ABCG1 and CCR7. These results indicate that SIRT1 is located upstream of LXRα signaling, which may be important for regulation of the development and progression of atherosclerosis.

The SIRT1-mediated activation of LXR may result in several positive effects in lipid metabolic and cardiovascular diseases, including the stimulation of cholesterol efflux from cells to high-density lipoproteins through the ABC transporters, ABCA1 and ABCG1, activating the conversion of cholesterol to bile acids in the liver and facilitating excretion (27). Therefore, we hypothesize that SIRT1-LXR-ABCA1/ABCG1 is one of the signaling pathways involved in the regulation of cholesterol efflux and is inhibited during atherosclerosis formation and progression, leading to cholesterol loading in macrophages and subsequent foam cell formation. In a mouse model of regression, rapid loss of plaque foam cells was observed to be due to migration to lymph nodes, a process reminiscent of dendritic cells. CCR7 is considered an essential factor for dendritic cell migration (6). Results of the present study indicate that CCR7 expression was enhanced, in addition to an increased expression of SIRT1 and LXR, which may promote foam cell migration from atherosclerotic plaques. These observations may represent a potential therapeutic target for the reversal of atherosclerotic plaques.

SIRT1 is known to physically bind the p65/RelA subunit of NF-κB and deacetylate p65 at lysine 310. This interaction downregulates the transcriptional activity of NF-κB, leading to a reduced inflammatory response (28). In the current study, NF-κB expression was enhanced in U937 cells by palmitate and Ox-LDL stimulation. In addition, activation of SIRT1 by SRT1720 suppressed NF-κB signaling. These results are consistent with previous studies which revealed that SIRT1-deficient macrophages exhibit NF-κB hyperacetylation, resulting in enhanced expression of various pro-inflammatory genes (28-30). In addition, results of the current study revealed that the SIRT1 inhibitor nicotinamide eliminated the effects of SRT1720, which restored the expression of inflammatory genes, including NF-κB, providing further evidence that SIRT1 functions upstream of NF-κB during foam cell formation.

Atherosclerosis is also a progressive chronic inflammatory disease (31). Active NF-κB is detected in aortae with clear atherosclerotic lesions; however, it is absent in normal, nonlesional aortae (32). A previous study by Gareus *et al* (33) demonstrated that inhibition of NF-κB abrogated the induction of adhesion molecules in endothelial cells, impaired macrophage recruitment to atherosclerotic plaques and reduced expression of cytokines and chemokines in the aorta of $ApoE^{-/-}$ mice. Thus, endothelial NF-κB signaling may orchestrate proinflammatory gene expression in the arterial wall and promote the pathogenesis of atherosclerosis. Inhibition of NF-κB activity is accompanied by a significant reduction in atherosclerotic lesion formation in $ApoE^{-/-}$ mice (33). The present study demonstrated that SIRT1 inhibited the expression and activity of the transcription factor NF-κB in foam cells, thus inhibiting the expression of proinflammatory factors, including TNFα and IL-1β. Therefore, in the process of monocyte-derived foam cell formation, the expression and activity of SIRT1 was suppressed, which may promote atherosclerotic plaque formation and progression by enhanced inflammation.

In summary, results of the present study indicate that downregulation of the LXR signaling pathway during foam cell formation is mediated by the suppression of SIRT1 in a high-triglyceride, high-cholesterol environment. When SIRT1 is activated, the expression of LXR and its target genes is upregulated, and these effects may be eliminated by the SIRT1 inhibitor nicotinamide. In addition, the suppression of SIRT1 may enhance inflammation by decreasing deacetylation of NF-κB and enhancing signaling. In conclusion, these results

demonstrate that SIRT1 may prevent atherosclerosis by enhancing the LXR-ABCA1/ABCG1/CCR7 and inhibiting the NF-κB signaling pathways.

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