Original Article
Overexpression of Sirt3 inhibits lipid accumulation in macrophages through mitochondrial IDH2 deacetylation

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Abstract: This study aims to explore the relationship between Sirt3 expression and lipid accumulation in macrophages by inducing mitochondrial IDH2 deacetylation. In this study, Sirt3 interference and overexpression lentiviral vectors were constructed. Macrophages collected from C57BL/6J mice by peritoneal lavage were used to construct Sirt3 gene interference and overexpression models, and cultured in medium containing 1 mg/ml ox-LDL for 72 h to observe the enrichment of ox-LDL. Reverse transcription PCR was used to detect the expression of Sirt3 mRNA, western blot to detect Sirt3 and acetylated IDH2 proteins, and Nile Red staining and flow cytometry to detect intracellular lipids in macrophages. The results indicated that as compared to Sirt3 overexpressed and normal groups, the acetylation of IDH2 and accumulation of ox-LDL were significantly higher in the Sirt3 inhibited group. In conclusion, the expression of Sirt3 can inhibit lipid accumulation in macrophages by inducing mitochondrial IDH2 deacetylation.

Keywords: Macrophages, mitochondria, Sirt3, IDH2

Introduction

Macrophages regulate lipid metabolism and inflammation by taking up large amounts of oxidized low-density lipoprotein cholesterol (ox-LDL) leading to intracellular lipid accumulation and formation of foam cells [1]. Monocytes in peripheral blood are induced by various cytokines inside blood vessel walls to differentiate into macrophages that are released to deal with the redundant lipid from ruptured foam cells. When more foam cells are formed and ruptured, this vicious cycle results in the formation of fatty necrotic matter, consisting of lipids, cholesterol crystals and cell debris [2]. Therefore, enrichment and metabolic disorders of lipids in macrophages is the major focus of atherosclerotic disease research and intervention.

Mitochondrial cholesterol transport is the key step to control the transport of cholesterol out of the macrophages [3], and is associated with the ATP combined transporters (ABC) [4, 5], which are critical molecules for cholesterol discharge [6-9], whose function is dependent on isocitrate dehydrogenase 2 (IDH2) of the Krebs cycle [10, 11]. Therefore, effective supply of energy is key for macrophage lipid processing. Additionally, the types of mitochondrial protein acetylation modifications include almost all the major metabolic pathways involved in mitochondrial proteins [12]. Therefore, we hypothesize that acetylation of IDH2 may affect the Krebs cycle.

Sirt3, a member of sirtuins (SIRTs), is a major regulatory enzyme in mitochondrial protein acetylation [13, 14]. Studies have shown that Sirt3 plays a role in the nucleus or the mitochondria based on cellular stress [15-17]. The regulation of Sirt3 gene in the liver, heart and skeletal muscle cells has been established [18-20]. However, its role in lipid accumulation in macrophages through inhibition of IDH2 acetylation remains unclear, and was examined in this paper.

Materials and methods

Animals

Male and female C57BL/6J mice were purchased from the Animal Center of Chongqing
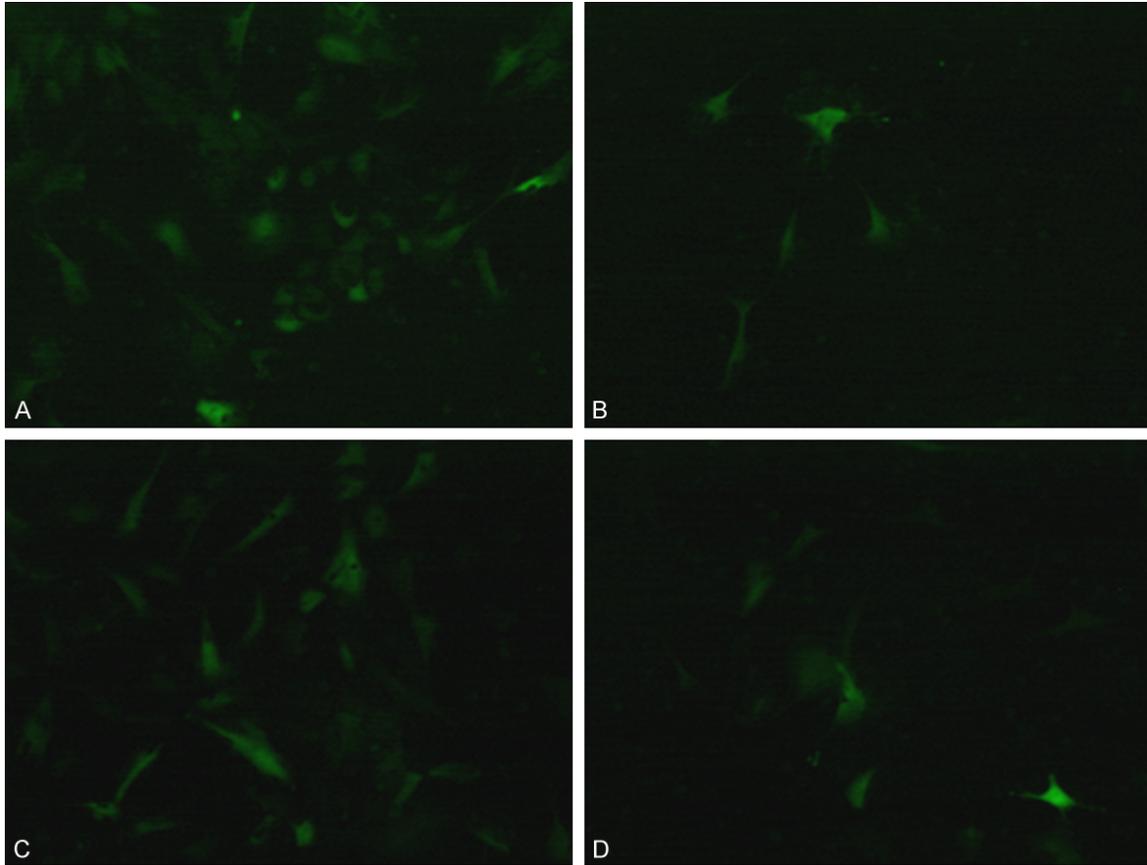
Figure 1. Images of macrophages and foam cells after transfection with Sirt3 interference and overexpression lentiviral vectors (100 ×). A. Sirt3 interference; B. Sirt3 interference cultured with ox-LDL; C. Sirt3 overexpression; D. Sirt3 overexpression cultured with ox-LDL.

Medical University, and individually housed. Details of the methods used to house and feed the mice have been previously described [21].

Preparation of cells

The macrophages were isolated from C57BL/6J mice by intraperitoneal lavage and cultured in 37°C, 5% CO_2 incubator for more than 24 hours to adapt to the environment. The cells were centrifuged and seeded at $3\text{-}5 \times 10^4$ cells/well in 24-well culture plates in RPMI1640 (Gibco, USA) with 100 U/ml each of penicillin and streptomycin (Huabeizhiyao, China) in 37°C, 5% CO_2 incubator.

Construction of Sirt3 siRNA lentiviral vectors for interference and overexpression

The Sirt3 sequence (NM_022433.2) of C57BL/6J mouse was obtained from Gene

Bank, and the target primer sequences for interference and overexpression were designed based on a previous study [22]. After ligation with GPH, the products were transfected into DH5α cells, and then identified by PCR and enzyme digestion. The positive clones were stored after sequence analysis.

Preparation of macrophages integrated with Sirt3 siRNA

After seeding the macrophages in 24-well plates for 24 hours, the Sirt3 siRNA lentiviral vectors were added to the wells for integration into the cells. Macrophages integrated with GPH lentiviral vectors only were used as the control group. After 24 hours, fresh culture medium containing 1 mg/ml ox-LDL (Sigma, USA) was added and the cells were cultured for 72 hours.
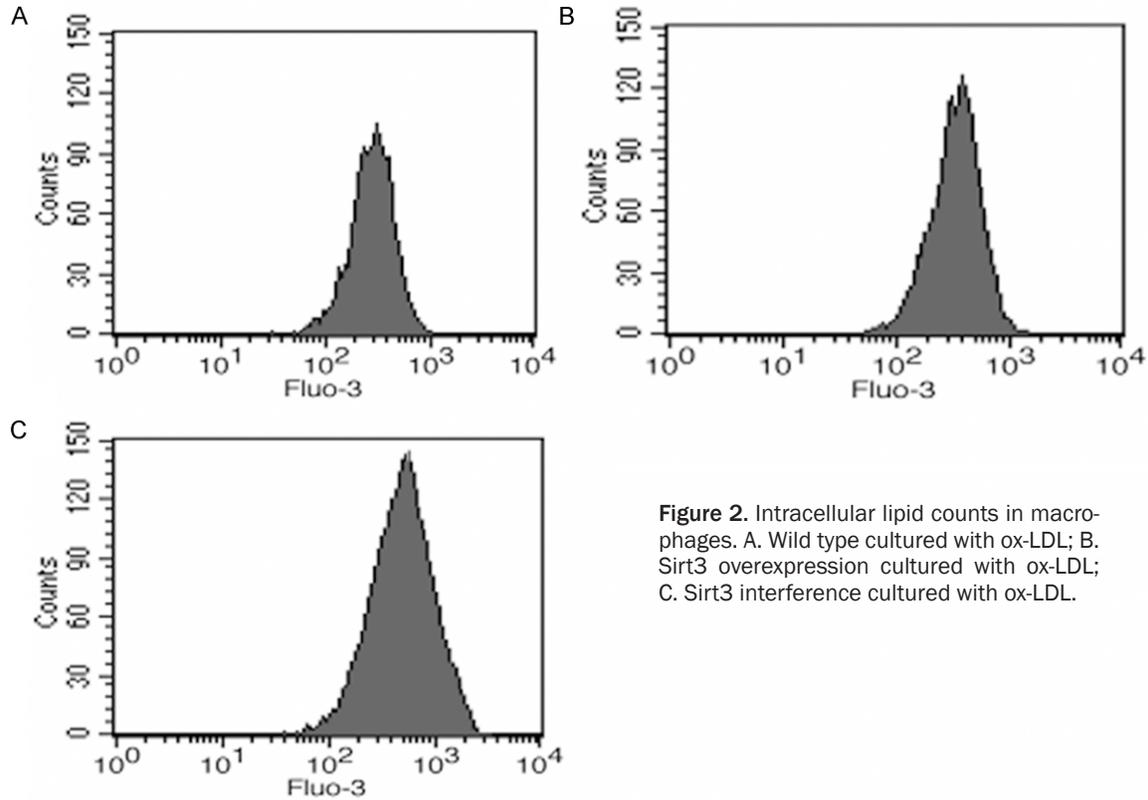
Figure 2. Intracellular lipid counts in macrophages. A. Wild type cultured with ox-LDL; B. Sirt3 overexpression cultured with ox-LDL; C. Sirt3 interference cultured with ox-LDL.

Flow cytometry detection

The concentration of ox-LDL in macrophages was analyzed using Nile red dye. Macrophages transfected with Sirt3 lentiviral vector were digested with trypsin (Sigma, USA), washed twice with PBS (Zhongshan, China), and 100 μl PBS and 10 μl Nile red dye (R&D, USA) were added. The cells were kept in a water bath at 37°C for 5 min, and then detected by flow cytometry.

Sirt3 mRNA detection

Sirt3 RNA was collected using TRIzol (ROCHE, Germany) from macrophages transfected with Sirt3 lentiviral vector and cultured in medium containing 1 mg/ml ox-LDL for 72 hours. The first-strand cDNA was synthesized using M-MuLV reverse transcriptase (dNTP Mixture M-MuLV Reverse Transcriptase, Promega), DEPC (Sigma, USA), and amplified by PCR (Taq, DNaseI, RNase free, TaKaRa). 8 μl of each PCR product was used for electrophoresis, with β-actin as the internal gene. The primers for Sirt3 were: GCTGCTTCTGCGGCTCTATAC and GAAGGACCTTC GACAGACCGT and for β-actin were: ATATCGCTGCGCTGGTCGTC and GCT CTC-CCTCACGCCATCCT.

Analysis of Sirt3 protein and acetylated IDH2

Antibodies used for western blotting included anti-IDH2 and anti-Sirt3 (R&D, USA). RIPA buffer contained 1 ml of 1.5 M NaCl (Guoyao Group), 5 ml of 100 mM Tris-HCl (pH 7.4) (Amresco, USA), 100 μl of 10% SDS (Bioins, Shanghai), 100 μl of 500 mM DTT (Amresco, USA), 100 μl of NP-40, complete mini protease inhibitors (Roche, Germany), and H_2O_2. For immunoprecipitation, macrophage mitochondrial lysates were incubated with anti-IDH2 and anti-Sirt3 antibodies overnight at 4°C. After resins were washed, samples were boiled with SDS loading buffer and subjected to western blotting [23] using GAPDH as the internal control.

The Labworks™ Analysis Software GDS8000 and UVP gel image processing system were used to quantitatively analyze the electrophoretic bands.
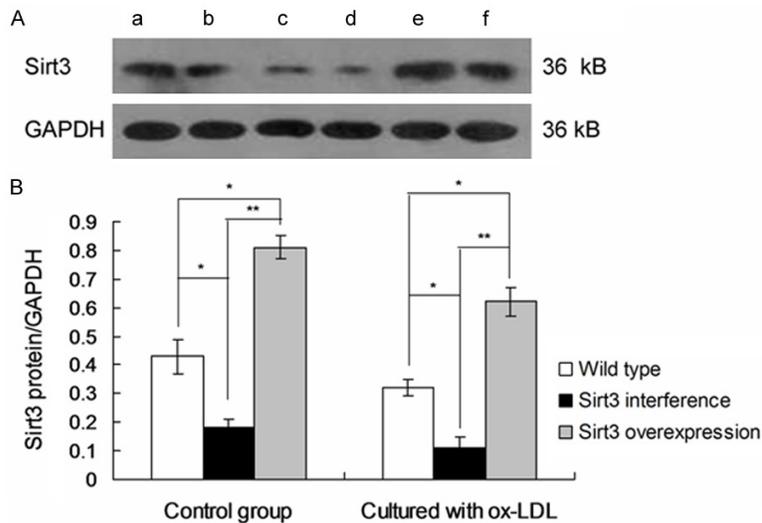
Figure 3. Observation of Sirt3 protein expression in macrophages by using Western blots. a. Wild type; b. Wild type cultured with ox-LDL; c. Sirt3 interference; d. Sirt3 interference cultured with ox-LDL; e. Sirt3 overexpression; f. Sirt3 overexpression cultured with ox-LDL. *P < 0.05, **P < 0.01 represent the comparison between group illustrated in images.
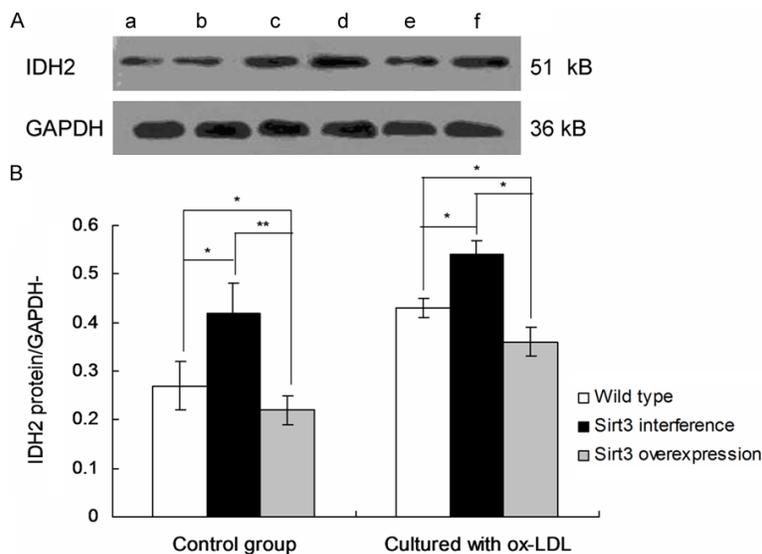
Figure 4. Observation of IDH2 acetylation in macrophages by using Western blots. a. Wild type; b. Wild type cultured with ox-LDL; c. Sirt3 interference; d. Sirt3 interference cultured with ox-LDL; e. Sirt3 overexpression; f. Sirt3 overexpression cultured with ox-LDL. *P < 0.05, **P < 0.01 represent the comparison between group illustrated in images.

Data processing and statistical analysis

All data were analyzed with SPSS (version 17) statistical package, and presented as mean ± SD. Two paired groups were compared using the *t*-test. P < 0.05 was considered to be statistically significant.

Results

Establishment of lentiviral vecrtor

Sirt3 interference lentiviral vector GPH-SIRT3-SH2 and overexpression lentiviral vector LV5-Sirt3 were successfully constructed with a titer of 3×10^8 TU/ml.

Ox-LDL triggers the adherence changes of Sirt3 treated cells

The lentiviral vectors were transfected into different groups of macrophages. The immunofluorescence images of macrophages are shown in **Figure 1**. The macrophages transfected with Sirt3 interference or overexpression lentiviral vectors cultured in medium containing ox-LDL, showed decreased adherence, which was more prominent in the latter than the former group (**Figure 1B** and **1D**).

Sirt3 inhibition induces the lipids accumulation

In order to understand this phenomenon, the intracellular lipids were detected by flow cytometry after Nile Red staining (**Figure 2**), which showed more lipids in Sirt3 inhibited macrophages than in wild type and Sirt3 overexpressed cells.

Sit3 inhibition increases the IDH2 acetylation and lipid accumulation

The Sirt3 protein expression and IDH2 acetylation were detected by western blot (**Figures 3** and **4**). The results showed that IDH2 acetylation and lipid accumulation in macrophages increased after inhibition of Sirt3 expression.

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Discussion

In this study, Sirt3 interference and overexpression lentiviral vectors were constructed and transfected into macrophages to study the influence of changes in Sirt3 expression on lipid accumulation and metabolism. Macrophages transfected with Sirt3 lentiviral vectors were cultured with ox-LDL to examine its intake and accumulation, and the generation of foam cells.

Immunofluorescence and flow cytometry confirmed that inhibition of Sirt3 expression increased the accumulation of ox-LDL in macrophages, while overexpression of Sirt3 increased the survival of macrophages. Accumulation of liposomes is an important means by which macrophages transform into foam cells [24-28]. Many foam cells formed by macrophages due to excessive cholesterol intake are seen in artery intima, which is a pathological sign of atherosclerosis [29].

Studies have found that Sirt3 is the strongest mitochondrial protein deacetylation enzyme due to its high deacetylation ability [30, 31], and IDH2 is its substrate in the TCA cycle [3-34], but whether this relationship exists in macrophages remains unknown. Our results found that mitochondrial acetylated IDH2 was significantly decreased when Sirt3 protein was overexpressed. After culturing with ox-LDL for 72 hours, the intracellular lipids and foam cells formation were lower in macrophages with Sirt3 overexpression than in macrophages with Sirt3 interference. Thus, changes in Sirt3 expression can regulate the accumulation of lipids and the formation of foam cells by altering the acetylation status of mitochondrial proteins in macrophages.

In conclusion, Sirt3 can inhibit lipid accumulation in macrophages by inducing mitochondrial IDH2 deacetylation.

Acknowledgements

Disclosure of conflict of interest

None.

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