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Original Article

Knockdown RIP-1 inhibits production of oxLDL-induced inflammatory cytokines and promotes ABCA1 expression in macrophages

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Abstract: *Background and objective:* Accumulation of oxidized cholesterol in macrophages is a key factor to initiate and accelerate atherosclerosis. In the present study, we investigated the role of receptor-interacting serine/threonine-protein kinase 1 (RIP-1, RIPK1) in the progression of oxidized low-density lipoprotein (ox-LDL)-induced inflammation of macrophages and the cholesterol transport outside of the macrophages. *Methods:* RAW264.7 cells were treated with increasing concentrations of oxLDL for indicated time. Inflammatory cytokines secretion was determined by ELISA assay. The levels of NF-κB p65, IκBα, RIP-1, TRAF3 and IKKα were determined by Western blotting analysis. *Results:* oxLDL induced inflammatory cytokines production in a time-and dose-dependent manner. The ox-LDL-induced cytokines production were mediated by NF-κB pathways in macrophage cells. oxLDL treatment upregulated the RIP-1 expression in RWA264.7 cells and interference with RIP-1 decreased the secretion of TNF-α and IL-6. Moreover, knockdown RIP-1 increased the ABCA1 levels in RAW264.7 cells. *Conclusion:* oxLDL promoted the expression of RIP-1 in RAW264.7 cells. Knockdown RIP-1 in macrophages decreased the inflammatory cytokines production and promoted the expression of ABCA1. Thus, inhibiting the level of RIP1 might be useful to suppress the chronic inflammatory response and promote the reverse transport of cholesterol in development of atherosclerosis.

Keywords: RIP-1, inflammatory, NF-κB, oxLDL, ABCA1

Introduction

Atherosclerosis is the major cause of morbidity and mortality of cardiovascular disease [1, 2]. It is a chronic inflammatory disease of the inner arterial wall and usually causes plaques formation of arterial wall involving in the innate and adaptive immune responses [3]. The formation of atherosclerotic plaque involves different innate immune cell types, such as monocytes, neutrophils, mast cells, dendritic cells and macrophages etc. [4, 5]. Innate immune response initiates the activation of nuclear factor-κB (NF-κB) signaling pathway, leading to the expression of a wide array of inflammatory cytokines [6, 7]. Recently, the relationship between innate immune response and the formation of atherosclerosis caused much attention [8, 9]. It has been found the macrophages in adipose tissue of patients with peripheral arterial disease produce more cytokines, such as tumor necrosis factor-alpha (TNF-alpha), monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6), which are involved in the progression of atherosclerosis [10-12].

The important role for the innate immunity in the development of atherosclerosis is due to the mediation of chronic inflammation, which eventually leads to the formation of foam cells [13, 14]. Recent evidences have demonstrated that Toll-like receptor, such as TLR4, TLR2 or MyD88, plays an important role in the development of atherosclerosis [13, 15]. Several endogenous signals such as oxidized low-density lipoproteins (ox-LDL) and exogenous signals

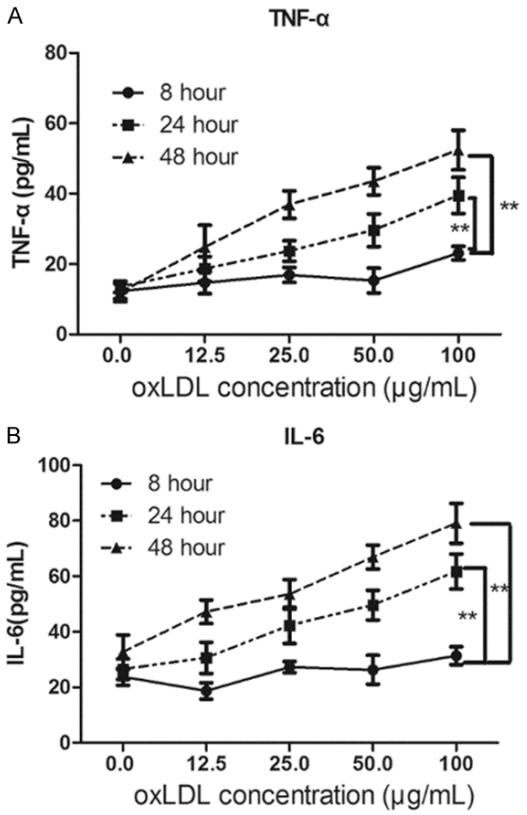

Figure 1. OxLDL induces the cytokines production of Raw264.7 cells. Raw264.7 cells were treated with 12.5, 25.0, 50 and 100 μg/mL of oxLDL and cultured for 8, 24 and 48 hours. The inflammatory cytokines TNF-α (A) and IL-6 (B) were determined by ELISA assay.

such as lipopolysaccharides (LPS) trigger pro-inflammatory responses and contribute to the formation of foam cells, which are considered to be key factors of initiating and accelerating atherosclerosis [16, 17].

Besides, atherosclerosis is also characterized by the disorder of lipids metabolism [18]. It has been demonstrated that cholesterol reverse transport is closely associated with adenosine triphosphate binding cassette transporter A1 (ATP-binding membrane A1, ABCA1), ABCG1 and scavenger receptor B1 (SR-B1) [19, 20]. Reverse cholesterol transport involves in transport of cholesterol back to liver for excretion and removal of the redundant cholesterol in peripheral tissues [21]. It is initiated by the cellular cholesterol efflux to lipid-free apolipoprotein A1 (ApoA1) or other lapidated high-density lipoprotein (HDL) particles, which is facilitated by ABCA1 and ABCG1 [22]. In the present study, we used oxLDL as stimulator to treat mouse macrophages and investigated the variation of key protein in the inflammatory response signaling pathway and RCT process. This study would give some new clues on the therapy of atherosclerosis.

Material and methods

Cell line and agents

Raw264.7 cells (Cat. No. CX0260) was purchased from Boster Co. (Wuhan, China). The cells were cultured in DMEM medium containing 10% FBS. Fetal bovine serum (Cat. No. 16000069) was purchased from Life Technologies (Invitrogen, USA). RIPK1 trilencer-27 mouse siRNA (Catalog No. SR418761) and Trilencer-27 universal scrambled negative control siRNA (Catalog No. SR30004) were purchased from Origene Corporation (Beijing, China). RNAse free siRNA duplex resuspension buffer (Catalog No. SR30005) was obtained from Origene Corporation. OxLDL was purchased from LuWenBio Corporation (Shanghai, China) and the catalog number was LW-6002.

ELISA assay

RAW264.7 cells (2×10^5 cells/mL) were plated into 48-well plate and treated with 12.5 μg/mL, 25.0 μg/mL, 50 μg/mL and 100 μg/mL of oxLDL for 8, 24 and 48 hours, respectively. The supernatants were harvested for detecting the concentration of inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) with ELISA kits (Neobioscience, Beijing, China) according to kit protocols, using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). The samples were analyzed in duplicate for cytokine levels.

Transfection

Raw264.7 cells were plated into 24-well plate and cultured to 70%-90% confluent for transfection. The transfection agent was lipofectamine TM 3000 regent (Catalog No. L3000001, ThermoFisher Scientific). The RAW-264.7 cells were transfected according to kit protocols. Briefly, 0.75 μL of lipofectamine 3000, 1 μ 3000, RIP-1 siRNAs or scrambled siRNAs were diluted with 50 μL of opti-MEM medium and mixed well with opti-MEM medium, respectively. Then, the diluted RIP-1 siRNA and scrambled siRNA was added to each tube of diluted lipofectamine 3000 regent (1:1 ratio). The mixture was incubated for 15 min at room

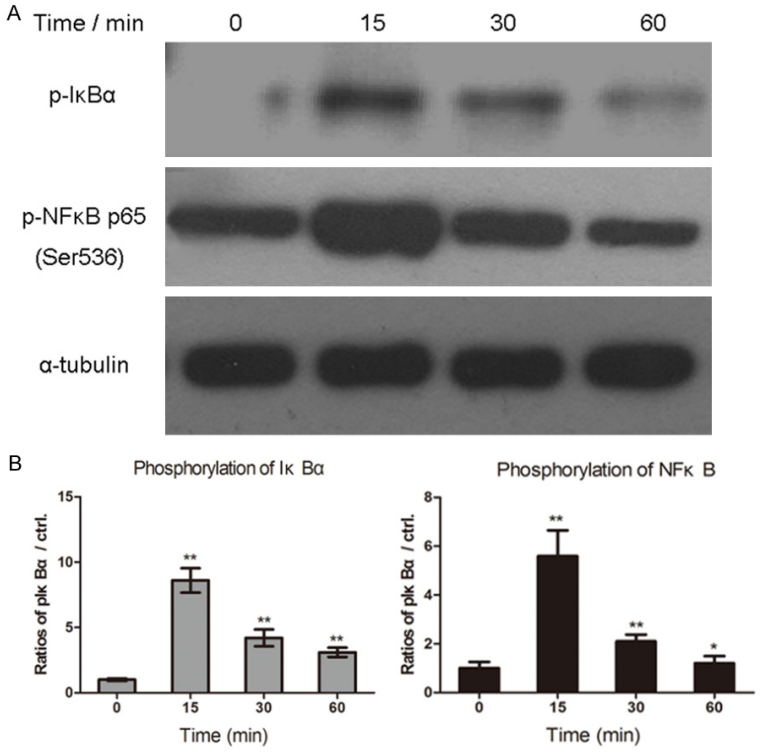

Figure 2. OxLDL activated NF-κB pathways in macrophage cells. A. Raw264.7 cells were treated with 50.0 μg/mL of oxLDL and the phosphorylation of IκB and NF-κB was tested by Western blotting in 15 min, 30 min and 60 min. B. The gray value of phosphorylated IκBα and NF-κB p65 subunit was shown in histogram. *p<0.05, **p<0.01, compared with control cells.

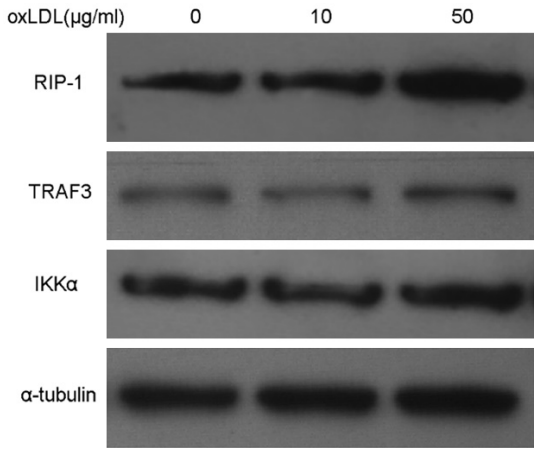

Figure 3. OxLDL treatment upregulates the expression of RIP-1. Raw264.7 cells were treated with 10 μg/mL and 50 μg/mL of oxLDL for 24 hours. The expression of RIP-1, TRAF3 and IKKα was determined by western blotting analysis. A-tubulin was used as internal reference gene.

temperature. Then, the siRNA-lipid complex was added to cells. The transfected cells were cultured for indicated time for other experiments.

Western blot

RAW264.7 cells were treated with different concentrations of oxLDL for indicated time. The cells were harvest and lysed by RIPA buffer (Catalog No. P0013B, Beyotime, Shanghai, China). The total protein was separated by SDS-PAGE and transferred at the condition of 400 m A for 2 hours. The primary antibodies used here were as follows:

The antibody of p-IκB-α Antibody (B-9) was a mouse monoclonal IgG provided at 200 μg/mL and purchased from Santa Cruz corporation. Anti-NF-κB p65 antibody (ab17742) was purchased from Abcam corporation, and it was a rabbit polyclonal to NF-κB p65. α-tubulin antibody #2144 was a rabbit polyclonal antibody and purchased from Cell signaling technology. RIPK1/RIP1 Antibody (Catalog No. NBP1-770-77) was a rabbit polyclonal, which was purchased from Novus Biologicals. Anti-TRAF3 antibody (ab-62552) was a rabbit polyclonal to TRAF3 and obtained from Abcam. Anti-IKK alpha antibody [Y463] (ab32041) was a rabbit monoclonal antibody [Y463] to IKK alpha and obtained from Abcam. β-catenin antibody (Cat. No. ab-16051) was obtained from Abcam Corporation.

Statistical analysis

The data was analyzed by SPSS 19.0 software. The concentration of inflammatory cytokines was analyzed by one-way ANOVA, and the other data was based on Student's *t* test. The data was shown as mean value ± S.D. P<0.05 was considered as statistically different.

Result

OxLDL induces the cytokines production of Raw264.7 cells

Excessive uptake of oxidized low-density lipoprotein (ox-LDL) induced the macrophage cells to form macrophage foam cells which was mediated via the scavenger receptors (SRs).

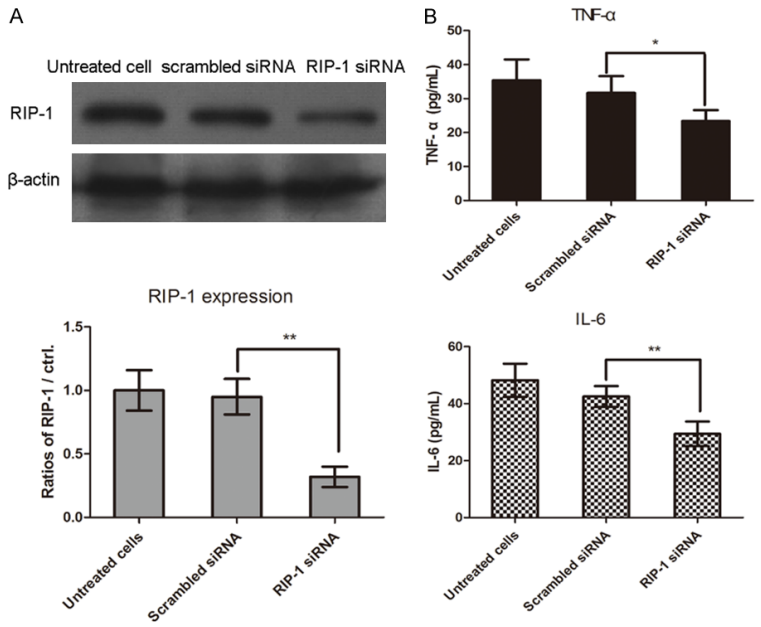

Figure 4. Interference with RIP-1 decreases the production of inflammatory cytokines in Raw264.7 cells. A. Raw264.7 cells were transfected with RIP-1 siRNA and scrambled siRNA for 24 hours and the expression of RIP-1 was tested by western blotting analysis. The gray value of RIP-1 level in RIP-1 siRNA-transfected cells or scrambled siRNA-transfected cells was shown in histogram. **p<0.01, compared with scrambled siRNA-transfected cells. B. The RIP-1 siRNA-transfected cells, scrambled siRNA-transfected cells and untreated cells were treated with 50 μg/mL of oxLDL for 24 hours and the secretion of TNF-α and IL-6 was determined by ELISA assay. *p<0.05, **p<0.01, compared with scrambled siRNA-transfected cells.

We firstly tested the production of inflammatory cytokines in mouse macrophage cells Raw 264.7. Raw264.7 cells were treated with 12.5, 25.0, 50 and 100 μg/mL oxLDL for 8, 24 and 48 hours, respectively. As shown in **Figure 1**, the inflammatory cytokines, including TNF-α and IL-6, showed increased production in a time- and dose-dependent manner. The results demonstrated that uptake of oxLDL by Raw264.7 cells obviously induced the production of inflammatory cytokines.

OxLDL activates NF-κB pathways in macrophage cells

In order to identify whether oxLDL activated NF-κB pathways to promote cytokines production, Raw264.7 cells were treated with 50.0 μg/mL oxLDL and the phosphorylation of IκB and NF-κB was determined by Western blotting. As shown in **Figure 2**, the phosphorylated IκB and NF-κb were significantly upregulated in 15 min and gradually decreased in 30 min and 60 min after oxLDL treatment in Raw264.7 cells. All the data obviously showed that the NF-κb signaling pathway was activated in ox-LDL-treated Raw264.7 cells, which contributed to the production of inflammatory cytokines.

OxLDL treatment upregulates the expression of RIP-1

Next, we wanted to know how the NF-κB pathway was activated by oxLDL in Raw264.7 cells. The levels of regulatory proteins in the upstream of NF-κB signaling were tested by Western blotting analysis. Raw264.7 cells were treated with 10 μg/mL and 50 μg/mL of oxLDL for 24 hours. As shown in **Figure 3**, the expression of RIP-1 was obviously upregulated in 50 μg/mL of ox-LDL-treated cells. However, the level of TRAF3 and IKKα was not obviously changed in ox-LDL-treated Raw264.7 cells, suggesting RIP1 might be a key regulator in oxLDL-induced secretion of inflammatory cytokines.

Interference with RIP-1 decreases the production of inflammatory cytokines in Raw264.7 cells

In order to investigate whether RIP-1 expression was regulated by oxLDL, RIP-1 specific siRNAs were used to transfect Raw264.7 cells. As shown in **Figure 4A**, the expression of RIP-1 was significantly decreased in RIP-1 siRNA-transfected cells than that in scrambled siRNA-transfected cells (**p<0.01). Importantly, the RIP-1 siRNA-transfected cells, scrambled siRNA-transfected cells and untreated cells were treated with 50 μg/mL of oxLDL for 24 hours and the production of TNF-α and IL-6 was determined by ELISA assay. As shown in **Figure 4B**, the level of TNF-α and IL-6 was significantly decreased in RIP-1 siRNA-transfected cells than that in scrambled siRNA-transfected cells (*p<0.05 and **p<0.01, respectively).

Knockdown of RIP-1 increases the expression of ABCA1 in Raw264.7 cells

Macrophage ATP-binding cassette transporter A1 (ABCA1) is reported to promote cholesterol

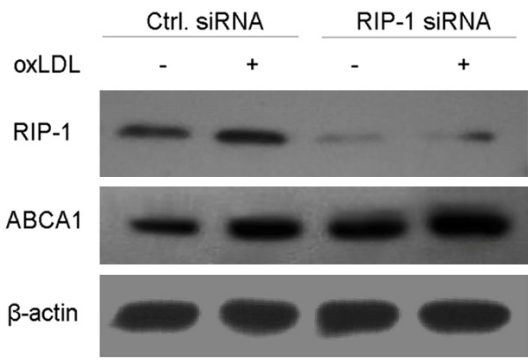

Figure 5. Knockdown of RIP-1 increases the expression of ABCA1 in Raw264.7 cells. The cells were transfected with RIP-1 siRNA and scrambled siRNA for 24 hours and were treated with or without 50 μg/mL of oxLDL for 24 hours. The expression of RIP-1 and ABCA1 was determined by western blotting analysis. Here, scrambled siRNA was used as negative control siRNAs.

efflux to extracellular matrix, called reverse cholesterol transport. We further investigated whether the expression of RIP-1 affected the macrophage cholesterol transport *in vitro*. As shown in **Figure 5**, oxLDL treatment increased the expression of RIP-1 and ABCA1 in Raw264.7 cells, and in RIP-1 siRNA-transfected cells, oxLDL obviously induced the expression of ABCA1 in Raw264.7 cells. All the data suggested that knockdown the level of RIP-1 increased the expression of ABCA1, which contributed to the cholesterol reverse transport into extracellular matrix and prevented the formation of macrophage foam cells.

Discussion

Atherosclerosis is normally considered as both symptoms of a chronic inflammatory responses and a lipid metabolism disorder [23]. Substantial evidence proves that oxidized LDL exerts a crucial role in the early inflammation responses and late atherosclerotic lesion [24]. Ox-LDL can trigger and promote mononuclear cell infiltration and the accumulation of oxidized cholesterol is one of factors to influence the formation of foam cells, while atherosclerosis is affected by multiple stresses [25]. In the present study, we used RAW264.7 cells as cell model and increasing concentrations of oxLDL were used to treat RAW264.7 cells. The inflammatory cytokines, including TNF-α and IL-6 was obviously up-regulated with the increasing concentrations of oxLDL. Moreover, the macrophages were treated with oxLDL for 8, 24 and 48 hours, respectively, and the results demonstrated that inflammatory cytokines were gradually increased with the increased time. All the data revealed that oxLDL treatment promoted the inflammatory response of macrophages, which could promote the foam cells formation from the macrophages.

Next, we found oxLDL-induced inflammatory cytokines production was mediated by the activation of NF-κB signals. Normally, NF-κB was inactive by binding with the inhibitory proteins of NF-κB and located in cytoplasm. When the activator occurs, IκB is phosphorylated and degraded, the NF-κB translocates into nucleus from the cytoplasm and targets to the specific sequence of the DNA to initiate the gene transcription. We tested the levels of RIP-1, TRAF3 and IKKα by Western blotting analysis and the results demonstrated that RIP-1 was obviously increased in 50 μg/mL of ox-LDL-treated macrophages. Importantly, knockdown of RIP-1 could significantly decrease oxLDL-induced inflammatory cytokines production. All the results demonstrated that RIP-1 mediated the oxLDL-induced inflammatory cytokines production.

Furthermore, we wanted to clarify whether RIP-1 regulated the reverse cholesterol transport in macrophages. We used RIP-1 specific siRNAs to transfect RAW264.7 cells and used scrambled siRNAs as negative controls. Western blotting was performed to test the ABCA1 expression in RIP-1 siRNA-transfected RAW-264.7 cells and scrambled siRNA-transfected Raw264.7 cells. The results obviously revealed that knockdown of RIP-1 increased the expression of ABCA1, suggesting knockdown of RIP-1 would significantly promote the reverse cholesterol transport and probably inhibit the formation of foam cells.

Thus, knockdown of RIP-1 in macrophages inhibited ox-LDL-induced inflammatory responses and promoted reverse cholesterol transport, which could probably be helpful to inhibit the progression of atherosclerosis.

Disclosure of conflict of interest

None.

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