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Knockdown of LSD1 meliorates Ox-LDL-stimulated NLRP3 activation and inflammation by promoting autophagy via SESN2-mesiated PI3K/Akt/mTOR signaling pathway

Xiaozhen Zhuo, Yan Wu, Yanjie Yang, Li Gao, Xiangrui Qiao, Tao Chen*

Department of Cardiovascular Medicine, First Affiliated Hospital of Xi'an Jiaotong University, China

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ABSTRACT

Aims: To explore the mechanism of how LSD1 regulates autophagy and the correlation between LSD1 and Ox-LDL-induced inflammation.

Main methods: RAW264.7 cells were used during the whole study. Firstly, the effect of Ox-LDL-stimulation on LSD1 expression was detected. Through loss-of-function assay, the associations between LSD1 interference and SESN2 expression, autophagy, NLRP3 inflammasome and inflammatory cytokines were explored. Finally, the function of LSD1 exerted on activation of PI3K/Akt/mTOR signal pathway was detected using western blotting assay.

Key findings: The expression of LSD1 was significantly elevated in Ox-LDL-treated RAW264.7 cells. Inhibition of LSD1 promoted autophagy, inhibited inflammation and activated NLRP3 inflammasome. SESN2 was elevated by LSD1 inhibition, and thus activate the PI3K/Akt/mTOR signal pathway. What' more, Knockdown of SESN2 or deactivate the PI3K/Akt/mTOR signal pathway partly reversed the effect of LSD1 inhibition on autophagy.

Significance: Our present study drew the finding that the knockdown of LSD1 meliorated Ox-LDL-stimulated NLRP3 activation and inflammation through promoting autophagy via SESN2-mediated PI3K/Akt/mTOR pathway.

1. Introduction

Cardiovascular diseases are high-incidence and high-risk human diseases, which contributed to a one-third mortality rate [1]. Atherosclerosis, as a most common member of cardiovascular diseases, always results in pernicious events such as ischemic stroke, myocardial infraction and even death, progressively [1,2]. Considering the characteristics of having a hand with inflammatory cells (mainly macrophages) and inflammatory cytokines in all stages, atherosclerosis is also regarded as a kind of chronic inflammatory disorder [3,4]. Thus, in order to clearly understand the related pathogenic mechanisms and search for effective treatment strategy of atherosclerosis, accumulating researches have devoted to demonstrate the crucial role of inflammatory stress. There was study proved that oxidized low-density lipoprotein (ox-LDL) was a main factor which contributed to the development and progression of atherosclerosis; from the mechanical view, it triggered monocyte to differentiate into macrophage, followed by the accelerated secretion of various inflammation-related mediators and corresponding cascades, thus to exert its pro-inflammatory nature [5–8]. In addition, a recent study provided Ox-LDL stimulation as a significant inducer of inflammatory response in RAW264.7 macrophages [9]. Moreover, ox-LDL was implicated in the thrombotic process via recruiting inflammatory cells and eliciting endothelial dysfunction in the early phase of atherosclerosis [10].

Inflammation is recognized as the foremost defense-line against injury or invading pathogen, for the homeostasis of our body [11]. Inflammasomes, the signaling complexes containing multi-protein, could accumulate in response to manifold stimuli, and then drive the progression of inflammation [11,12]. In general, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), the cysteine protease caspase-1 and one nucleotide-binding oligomerization domain-like receptor (NLR) protein were the typical components of a inflammasome [13]. Thereinto, NLR pyrin domain containing 3 (NLRP3) inflammasome came to our sight due to the implication of its activation in atherosclerosis-related inflammation [14]. There was evidence emerged that NLRP3, a particular member of NLR

* Corresponding author at: Department of Cardiovascular Medicine, First Affiliated Hospital of Medical School, Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi 710061, China.
E-mail address: chentao0331@xjtu.edu.cn (T. Chen).

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family, could be activated by multiple Damage-Associated Molecular Patterns (DAMPs) to form inflammasome, then triggered the accumulation of pro-IL-1β which followed by the induction of IL-1β release via Caspase-1 process [15]. Besides, the research of Duewell and his colleagues exhibited that ox-LDL could stimulate the NLRP3/IL-1β cascade to give rise to the furtherance of atherosclerotic inflammation [16].

In the exploration on the mechanism of the development and progression of atherosclerosis, researchers found that ox-LDL could not only cause the dysfunction in endothelial cells, but also activate autophagy in atherosclerosis-related cells [17,18]. It had been documented that autophagy dysregulation could result in pathophysiological processes of various cardiovascular diseases, atherosclerosis included [19,20]. Also, autophagy dysfunction was found in the developed atherosclerotic plaque [21]. Besides, previous study demonstrated that autophagy suppressed the formation of pro-inflammatory complex and efficiently clear the damaged organelle [22]. The emerging evidence indicated that autophagy, a protective metabolic process, had an apparent association with atherosclerosis and was becoming an essential focus of atherosclerosis prevention. A recent study proved that macrophage played a driven role in atherosclerosis progression [23]. Even for all these, the exact correlation and function mechanism of autophagy and atherosclerotic inflammation in ox-LDL-induced macrophages were still indistinct.

Recently, lysine-specific demethylase 1 (LSD1), the first identified histone demethylase, had been documented to have association with autophagy in tumor-related studies. Cited as an instance, LSD1 knockdown functioned as a promoting role on both autophagy activation and proteasomal activity along with inhibited ubiquitin factor E4B [24]. The investigation of Chao et al. concluded that LSD1 repressed autophagy processing in gynecologic malignancies [25]. However, we didn't get clearly informed of the part LSD1 played in atherosclerosis-related inflammation. Moreover, SESN2 (sestrin 2), a member of the highly conserved sestrin family, played a protective part against various insults including oxidative stress and DNA damage and was proved as a mediator of autophagy in tumorigenesis regulation which was targeted by LSD1 [26–28]. There was evidence indicated that stimulating the expression of SESN2 acted an advantageous role to ox-LDL stress for the survival of RAW264.7 cells [29]. More interestingly, a study focused on myocardial infarction suggested that SESN2 suppressed the inflammatory responses via activated macrophages, classically [30]. Therefore, our present study speculated that LSD1 might play a part in the regulation of autophagy and atherosclerotic inflammation with the involvement of SESN2.

In the present study, we investigated the effect of ox-LDL stimulation on the expression of LSD1 and explored how LSD1 acted on ox-LDL-stimulated NLRP3 inflammasome activation and inflammatory response. Finally, we confirmed the relation between the LSD1, autophagy and inflammation development, which might provide a potential therapy target and strategy for atherosclerosis prevention.

2. Materials and methods

2.1. Cell culture and drug administration

American Type Culture Collection (ATCC, Manassas, VA, USA) was the supplier of RAW264.7 murine macrophage cell line used in this study. Fetal bovine serum was treated under 65 °C for 20 min heat-inactivation and formulated into 10%. Then, we supplemented the prepared fetal bovine serum into Dulbecco's Modified Eagle Medium (DMEM), which contained with sodium pyruvate, stable glutamine and high glucose, along with 1% antibiotics, 100 IU/mL penicillin and 100 mg/mL streptomycin. Subsequently, RAW264.7 cells were plated into T-75 flasks those filled with the specific DMEM, and cultured in a 5% CO_2 setting HeraCell CO_2 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the temperature of 37 °C. It is noteworthy that the medium should be changed every 48 h.

The reagents used in the present study were ox-LDL (Merck KGaA, Darmstadt, Germany), baflomycin A1 (Abcam plc., Cambridge, MA, USA) and 740Y-P (MedChemExpress, Monmouth Junction, NJ, USA), and every reagent was diluted into target concentrations according to the requirement of the experimental design. With regard to the processing method, we implemented baflomycin A1 and 740Y-P administration (both 24 h) after transfection assay, on top of that ox-LDL treatment was followed. Besides, dimethyl sulfoxide (Solarbio® life science, Beijing, China) was set as control of drug treatments.

2.2. RT-qPCR

Total RNA extraction of the cultured cells was performed after all transfection and reagent treatments had been completed, by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) in line with its manufacturer's protocol. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was the commercial kit which we utilized for the synthesis of cDNA from the purified RNA, and the reverse transcription reaction was executed on a PCR platform, ProFlex™ 3 × 32-well PCR System (Applied Biosystems, Foster City, CA, USA). Subsequently, the amount of mRNA expression of target gene in the conditioned cells was measured on ABI 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using Fast SYBR® Green Master Mix (Invitrogen, Carlsbad, CA, USA). The specific primers were designed by the online software Primer 6.0 (Premier, Canada) synthesized by Invitrogen (Table 1; Carlsbad, CA, USA). Apart from that, the minute qPCR procedure was described as below: 1 min at 95 °C, 20 s at 95 °C and 10 s at 56 °C and 15 s at 72 °C for 35 cycles, finally held at 4 °C. According to $2^{-\Delta\Delta Ct}$ method, the relative abundance of gene expression was assessed at last.

2.3. Western blotting

For the determination of cellular protein expression in this study, the following primary antibodies were purchased from Cell Signaling Technology Inc. and were diluted at 1:1000 (Danvers, MD, USA): anti-LSD1 (#2139), anti-SESN2 (#8487), anti-LC3(#4108), anti-p62 (#5114), anti-NLRP3 (#15101), anti-ASC (#67824), anti-Caspase-1 (#4228), anti-PI3K (#4257), anti-p-PI3K, anti-Akt (#2920), anti-p-Akt (#4060), anti- mTOR (#2972), anti-p-mTOR (#5536) and GAPDH LSD (#5174). Synchronously, we obtained the species-specific horseradish peroxidase-conjugated secondary antibody from the same company with its dilution of 1:5000. According to the instruction of manufacturers, we prepared protein extract by RIPA lysis buffer (CW Biotech, Beijing, China), and the protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). After separation of equal amounts of protein lysates (50 μg) through 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, we transferred the proteins onto polyvinylidene difluoride

Table 1
Primer sequences used in reverse transcription quantitative polymerase chain reaction.

Gene	GenBank accession number	Primer sequence (5′ → 3′)
LSD1	NM_001347221.1	F: AGCTTCTAGAGGATCCACTAGT R: AGCTTCTAGACTCGAGCGGCCG
SESN2	NM_144907.1	F: AGAGGGCACAGGAAAGAA R: TCAAGCATAAAGGACCAAA
TNF-α	NM_013693	F: CAGCCTCTTCTCCTTCCTGA R: GGAAGACCCCTCCCAGATAGA
IL-6	NM_031168.2	F: AACGATGATGCACTTGCAGA R: GAGCATTGGAAATTGGGGTA
IL-1β	NM_008361.4	F: TTCAACACGCAGGACAGGTACAG R: CCAGGGACAGGATATGGAGCA
GAPDH	NM_008084.2	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA

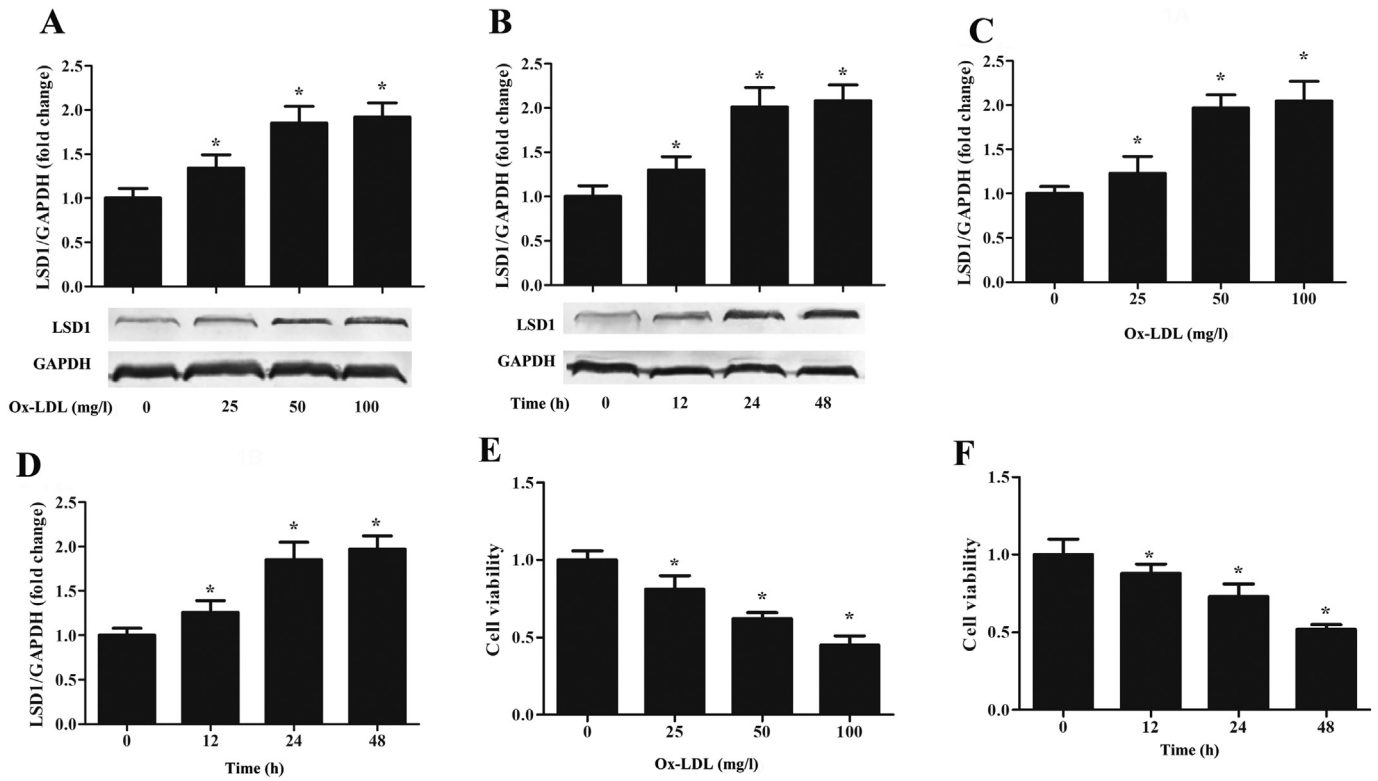

Fig. 1. Ox-LDL upregulates the expression level of LSD1 in RAW264.7 cells. The cultured RAW264.7 cells used in this section were divided into two groups. One group was treated with different concentrations (0 mg/L, 25 mg/L, 50 mg/L and 100 mg/L) of ox-LDL for 24 h, the other was used for the treatment of 50 mg/L ox-LDL for the indicated times (0 h, 12 h, 24 h and 48 h). Next, the cells treated according to the experimental design were used to determine the expression level of LSD1 by RT-q PCR and Western blot assay. (A) ox-LDL treatment apparently elevated the protein expression level of LSD1 in dose-dependent manner. (B) The fold change of LSD1 protein expression in cells which treated with ox-LDL for different times was determined by western blotting. (C and D) The results of RT-qPCR showed the effects of different LDL concentrations (C) and treating times (D) on LSD1 transcription in RAW264.7cells. (E and F) The results of CCK-8 showed the effects of different LDL concentrations (E) and treating times (F) on cell viability in RAW264.7cells.The data was expressed by mean ± SEM. Six replicates for each group and the experiment repeated three time in the study. Differences between the groups were examined for statistical significance using one-way ANOVA. $^{*}P < 0.05$ versus 0 mg/L and 0 h, respectively.

membranes (Millipore, Billerica, MA, USA). Incubation with the primary antibodies was conducted on the membranes. Then, the complex was washed three times by PBST for the next secondary antibody incubation. Finally, the western blot bands were obtained from Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). It was necessary for setting GAPDH as the endogenous control to normalize protein expressions, and we used the fold change versus GAPDH group to present the relative target proteins' expression levels.

2.4. CCK-8 assay

Cells (5×10^4 well) were seeded into a 96-well plate and cell proliferation was measured using a CCK-8 kit (Vazyme, Piscataway, NJ, USA) according to the manufacturer's instruction, and the absorbance was detected on a microplate reader (490 nm).

2.5. Enzyme-linked immunosorbent assay (Elisa)

After reagent treatment or transfection experiments had been completed, we harvested and isolated cells by a 10 min centrifugation at 16,000 $\times g$ to get the supernatants which were used for the detection of various cytokines' expression levels. According to the protocol of Enzyme-linked Immunosorbent Assay (ELISA) Kit (eBioscience, Inc., San Diego., USA), the quantification of the detected results was analyzed. In addition, we developed the plates with tetramethylbenzidine substrate and used an absorbance at 450 nm obtained from BioTek ELx800 (BioTek, Winooski, VT, USA) to calculate the optical density. The antibodies used in this study, anti-TNF-α, anti-IL-6, anti-IL-1β and streptavidin-horseradish peroxidase, were supplied by Chemicon International (Billerica, MA, USA).

2.6. siRNAs and overexpression vector transfection

Cells were seeded at a density of 1.2×10^5 cells/well in a culture dish. siRNA interference assay was conducted when the conditioned cells were cultured reaching a confluence of 85%. In accordance with the manufacturer's recommended instruction of Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), the satisfactory cells were transfected by siLSD1 or siSESN2 which were synthesized by Invitrogen (Carlsbad, CA, USA) as the following sequences: siLSD1: FPS as 5′-CCACCGAGU UCACAGUUAUTT-3′ and RPS as 5′-AUAACUGUGAACUCGGUGGTT-3′. Overexpression plasmid LSD1 Flag-LSD1 plasmid was transfected into cells using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. The transfection reaction continued for 24 h, and then the solution was replaced with fresh complete medium for another 24 h incubation, prior to other drug or reagent treatments on the cells.

2.7. Statistical analysis

By the usage of online software Primer 6.0 (Premier, Canada) we designed all the primers, and all the primers and siRNAs were synthesized by Invitrogen (Carlsbad, CA, USA). We performed triplicate reactions repeated independently in the present experiment. For the comparison of difference significant between every two groups, two-tailed Student's *t*-test was applied, along with unpaired t-test way. An

ANOVA was also performed comparing more than two groups. The results were presented as a mean ± standard error of the mean. Besides, the SPSS software package (Statistical Package for the Social Sciences, version 17.0, SPSS Inc. Chicago, IL, USA) was utilized for the performance of statistical analysis. A *P* value below 0.05 was considered statistically significant.

3. Results

3.1. Ox-LDL upregulates the expression level of LSD1 in RAW264.7 cells

From the results of RT-qPCR and western blot assays, we concluded that ox-LDL stimulation significantly and dose-dependently up-regulated the experiment of LSD1, both in transcriptional and protein level; as well, 50 mg/L was the most appropriate concentration of Ox-LDL to elevated the expression of LSD1 to two fold changes (Fig. 1A and C). Besides, in the experiment of ox-LDL treatment on cells for indicated times, the incubated cells showed a gradually enhanced LSD1 protein expression while the treated time extended; however, we found that the increasing amplitude of LSD1 expression was declined when cells were treated for > 24 h (Fig. 1B). With regard to ox-LDL treatment times' effect on the mRNA expression of LSD1 in RAW264.7 cells, we had drawn a similar alteration tendency and proposed 24 h as the most efficient processing time (Fig. 1D). Besides, the cell proliferation was gradually decreased by Ox-LDL treatment in a dose and time dependent (Fig. 1D and E). Generally speaking, 50 mg/L ox-LDL treatment for 24 h had the most significant up-regulation effect on the expression of LSD1 in RAW264.7 cells.

3.2. LSD1 regulates autophagy in Ox-LDL-treated RAW264.7 cells

The results showed that the LSD1 siRNA silenced LSD1 and pcDNA/LSD1 overexpressed LSD1 effectively in ox-LDL-stimulated cells (Fig. 2A). The visualized and quantized results of western blot assay implied that ox-LDL obviously upregulated the expressions of autophagy-related genes, LC3II and p62; on this basis, the interference of LSD1 had reinforced this effect, while overexpression of LSD1 lowered the expressions of LC3II and p62, significantly (Fig. 2B and C). To sum up, our results gave the propose that interfered LSD1 could promote autophagy in ox-LDL-induced RAW264.7 cells.

3.3. Knockdown of LSD1 suppresses ox-LDL-stimulated inflammation by activating autophagy

According to the result of our detection on inflammatory cytokines, it was indicated that ox-LDL treatment significantly up-regulated the transcriptional levels of TNF-α, IL-6 and IL-1β, which were depressed by the interference of LSD1 and increased by LSD1 overexpression; however, the treatment of baflomycin A1 were both counteracting educed mRNA expression of these inflammatory cytokines induced by LSD1 siRNA in ox-LDL-stimulated cells with significant differences (Fig. 3A, B and C). The results of Elisa kits showed the same results as the expression level of respective mRNA (Fig. 3C, D and E). Taken together, the interfered-LSD1 played an inhibitory role in inflammation ox-LDL-stimulated cells.

Indeed, during the studies of cardiovascular disease-related inflammation, researchers had validated the pivotal role of NLRP3 inflammasome [11]. Combined with the aforementioned evidence, we implemented detections on the expressions of NLRP3 inflammasome related proteins. Consistent with the alteration of inflammatory cytokines, the expression of NLRP3 was increased by ox-LDL stimulation, and the subsequent knockdown of LSD1 depressed NLRP3 expression by a big margin; but, the downregulation of NLRP3 induced by LSD1 siRNA was reversed by baflomycin A1 (Fig. 4A and B). The other two key proteins of NLRP3 inflammasome, ASC and Caspase-1, also performed the same alteration tendency with NLRP3 under the treatments of ox-LDL, siRNAs and the inhibitor of autophagy, baflomycin A1 (Fig. 4A, C and D). Collectively, the conclusion might be drawn as: LSD1 played a regulatory part in NLRP3 inflammasome assemble in ox-LDL-stimulated cells.

3.4. LSD1 inhibition activated autophagy via SESN2-mediated PI3K/Akt/Mtor pathway

In the line with a recent study which concentrated on neuroblastoma, we learned that LSD1 could bind to the promoter of SESN2, and inhibition of LSD1 triggered SESN2 expression leading to the enhanced autophagy [27]. So it was indispensable to implement loss-of-function assay for the verification of the role SESN2 acted between LSD1 and autophagy under ox-LDL treatment. In like manner, SESN2 siRNA also suppressed the protein expression of SESN2 in the cells treated with ox-LDL effectively; except that, knockdown of LSD1

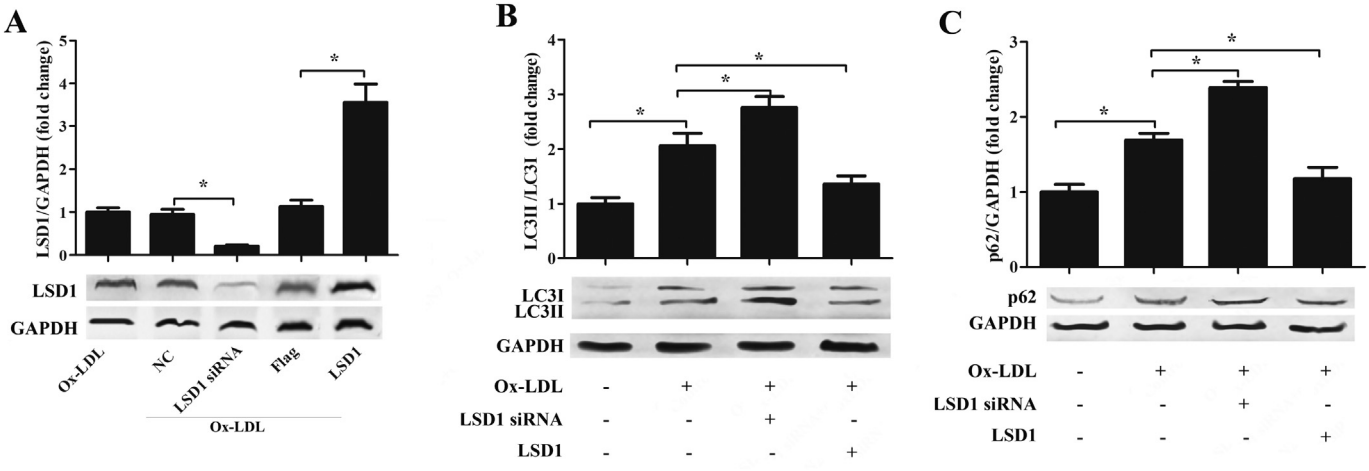

Fig. 2. LSD1 siRNA promotes autophagy via upregulation of SESN2. In the first place, cells were transfected by LSD1 siRNA or Flag-LSD1 plasmid at the concentration of 50 nM, respectively. Secondly, after removing transfection reagents, cells were treated with 50 mg/L ox-LDL for 24 h. At last, the protein expression level of LSD1, SESN2 and autophagy-related molecules were detected using western blotting. (A) LSD1 expression level was detected by western blotting. *$P < 0.05$ versus ox-LDL group. (B) The fold change of LC3II protein was visualized and quantized through western blot assay. (C) The expression of p62 was obtained from the execution of western blotting. Six replicates for each group and the experiment repeated three time in the study, and the results were expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA, * represents a *P* value < 0.05 between two groups in figure B, C and D.

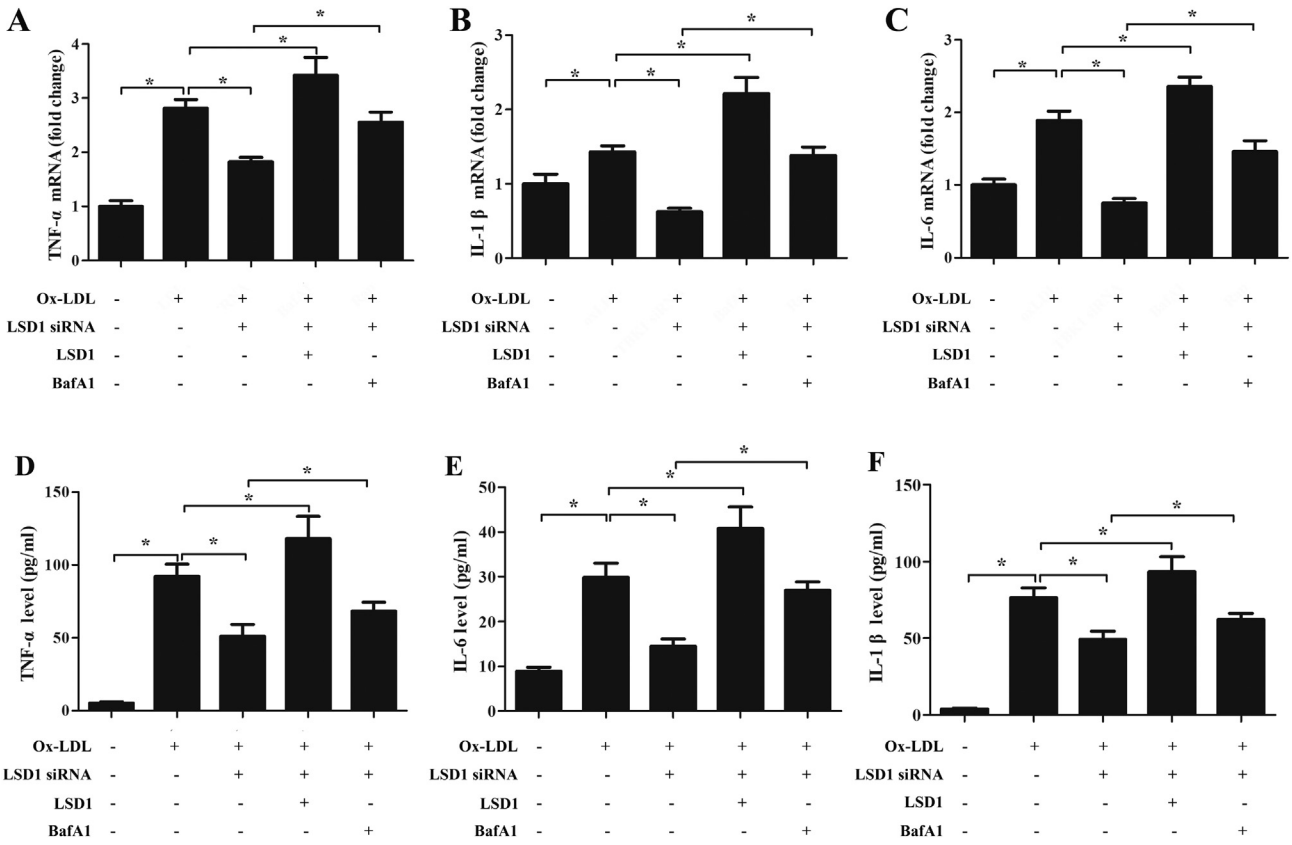

Fig. 3. Knockdown of LSD1 suppresses ox-LDL-stimulated inflammation. Transfection assay was implemented on the conditioned cells using 50 nM LSD1 siRNA or Flag-LSD1 plasmid. Then, a part of LSD1 siRNA-transfected cells was treated with 50 nM baflomycin A1. Eventually, ox-LDL at the concentration of 50 mg/L was utilized for a 24 h incubation on the treated cells. Afterwards, the target indicators of these cells were detected. (A, B and C) The fold change of mRNA levels of TNF-α (A), IL-6 (B) and IL-1β (C) were detected using RT-qPCR method. (D, E and F) Elisa assay was executed for the determination of the quantity of inflammatory cytokines in cells, TNF-α (D), IL-6 (E) and IL-1β (F). Six replicates for each group and the experiment repeated three time in the study, data was described as mean ± SEM. Statistical analysis was performed by one-way ANOVA, $^{*}P < 0.05$ between groups of comparisons.

significantly raised the promotion of ox-LDL brought to SESN2 expression (Fig. 2B). That is to say, LSD1 could modulate ox-LDL-induced SESN2 upregulation.

To further figuring out the mechanism of LSD1 mediated Ox-LDL-triggered autophagy and inflammation, the effects LSD1 inhibition on PI3K/Akt/mTOR pathway was analyzed. The results of western blotting showed that ox-LDL-stimulated cells significantly expressed higher phosphorylated PI3K p85, Akt and mTOR compared with those in control group; however, when the cells were transfected with LSD1 siRNA, the expression of these target genes had a notable decline tendency (Fig. 5A, B, C and D). Furthermore, the results indicated that 740Y-P and SESN siRNA performed as an inhibitor of the up-regulation of LC3II and p62 which induced by LSD1 siRNA in ox-LDL-stimulated cells (Fig. 5E and F). Summing up, our study validated that the suppression of LSD1 knockdown exerted on ox-LDL-induced autophagy was via SESN2-mediated activation of PI3K/Akt/mTOR pathway.

4. Discussion

In the manuscript of present study, we delineated the exploration of the potential mechanism of how LSD1 exerted on autophagy and inflammation along with their association, using an in vitro model of ox-LDL-stimulated RAW264.7 cells. The headmost finding of the current study was that ox-LDL significantly upregulated the expression level of LSD1. In line with this, the previous study confirmed the elevation of LSD1 under various pathological states [25]. Recent research indicated that the down-modulated LSD1 by genetic knockdown or pharmacological inhibition, induced the increase of LC3II and p62 proteins and the boost of autophagy [25,31]. Our findings here also suggested that the attenuation of LSD1 played a promoting role in Ox-LDL-induced autophagy. In the meantime, we got the evidence that LSD1 inhibition promoted SESN2 upregulation under Ox-LDL stimulation. Also, there was evidence that oxidative stress induced by hypoxic-ischemic encephalopathy obviously enhanced SESN2 expression [28]. And our finding was a matching one of a previous research, in which Ambrosio and colleagues validated that LSD1 could bind to the promoter region of SESN2 and repress its expression [27]. Besides, SESN2 were also reported to be responsible for autophagy regulation. For instance, SESN2 was demonstrated to participate in an autophagy-induced pathway protecting renal tubules during acute kidney injury [26,32].

In the light of studies on the pathway of regulating autophagy, the PI3K/Akt/mTOR signal pathway had been attested to have connection with autophagy in cardiovascular disease. As an example, the inactivation of PI3K/Akt/mTOR pathway was the hinge of kaempferol to alleviate Ox-LDL-induced apoptosis by up-regulating autophagy in human endothelium [33]. There was also a study suggested that the selective inhibition of PI3K/Akt/mTOR signaling pathway was a regulator of macrophage autophagy and stabilization of the vulnerable atherosclerotic plaques [34]. In the study by Yin et al., the down-regulation of PI3K/Akt/mTOR pathway promoted Ox-LDL-induced autophagy during atherosclerosis [35]. Furthermore, S2101, one of the most potent LSD1 inhibitor, exhibited the anticancer function by triggering autophagy and apoptosis along with the association of Akt/mTOR pathway depression [36]. At the same time, mTOR was found to be suppressed by p53-induced SESN2 upregulation, resulting in the induction of autophagy under various stress conditions [37]. For

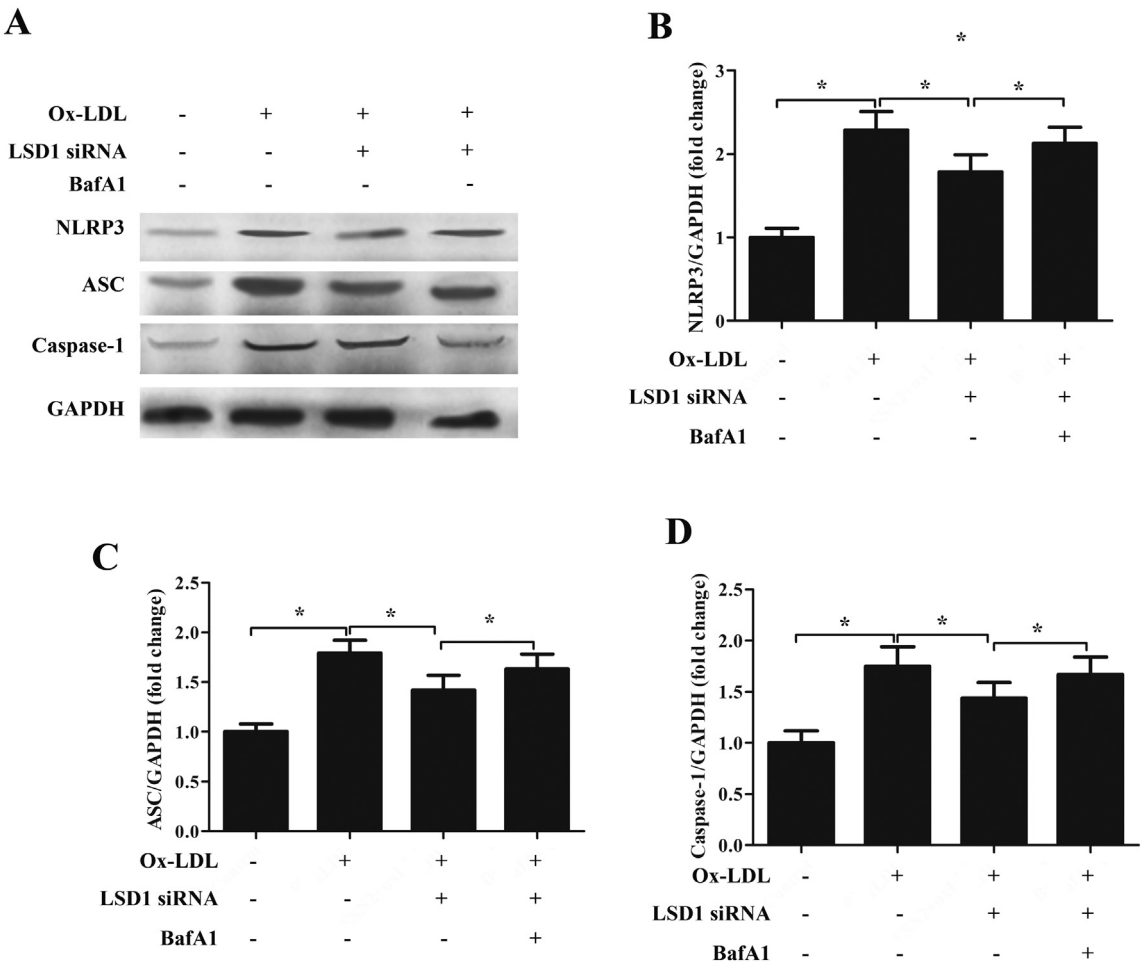

Fig. 4. LSD1 modulates NLRP3 inflammasome formation. RAW264.7 cells were firstly transfected by LSD1 siRNA for 24 h; the concentration of siRNA was 50 nM. 50 nM baflomycin A1 was only used to incubate partial cells which had been transfected by LSD1 siRNA, prior to being stimulated with 50 mg/L ox-LDL for 24 h. Subsequently, the NLRP3 inflammasome related proteins were tested. (A) The visualized protein bands were obtained by western blot assays and imaging device. (B, C and D) The protein expression fold changes of NLRP3 inflammasome-related cytokines, NLRP3 (B), ASC (C) and Caspase-1 (D), were detected using western blotting method. Statistical analysis was performed by one-way ANOVA, *P* value of groups' comparison which < 0.05 was delineated as *. Six replicates for each group and the experiment repeated three times in the study. All the data in this study was presented as mean ± SEM.

echoing the evidence, we executed corroboration on the participation of PI3K/Akt/mTOR pathway. And the results proved that LSD1 promoted the phosphorylation of PI3K, Akt and mTOR, and inhibition of SESN2 also contributed to the activation of the pathway. Generally, the inhibition of Ox-LDL-stimulated autophagy exerted by LSD1 was through SESN2-mediated PI3K/Akt/mTOR pathway activation.

Ox-LDL, oxidized from excessive LDL, was recognized as a main risk factor of atherosclerosis [8]. Apart from the crucial role it played in autophagy promotion, Ox-LDL had interaction with macrophages, endothelial cells and smooth muscle cells which involved in the development of atherosclerosis, and drove the secretion of inflammatory cytokines such like TNF-α, IL-6 and IL-1β [38–40]. Now that autophagy was a physiological process which played a protective part under stimuli had been proved, we reasonably proposed that autophagy might prevent inflammation in Ox-LDL-stimulated cells. During the exploration on the distinct mechanism of morin hydrate against inflammation in atherosclerosis, Zhou and colleagues indicated that autophagy was an important participator [41]. In addition, the induction of autophagy accelerated the activation of AMPK/mTOR pathway thus to result in the inhibition of inflammation in macrophages [42]. In our study, we verified the prevention function of autophagy exerted on Ox-LDL-induced inflammation. What's else, we further draw the conclusion that the defensive induction of autophagy was mediated by the SESN2-modulated passivation of PI3K/Akt/mTOR pathway which was regulated by LSD1.

Previously, NLRP3 inflammasome was proposed had a positive relation with atherosclerotic related inflammation. Besides, the reduced inflammation and lesions of atherosclerosis were marked in NLRP3 inflammasome inhibited tissues [43]. It was validated that the autophagy triggered in macrophages was a depressed way of berberine exerted on the activation of NLRP3 inflammasome [44]. The inhibition of autophagy was reported to induce the release of inflammatory cytokine partly through NLRP3 inflammasome activation [45]. Furthermore, SESN2 was demonstrated as a suppression role of NLRP3 inflammasome activation in macrophages [46]. In this research, we got a further consequence compared with the antecedent evidence. According to our understanding, our study demonstrated for the first time that LSD1 could regulate NLRP3 inflammasome assembly through SESN2-mediated autophagy, thus acting on Ox-LDL-stimulated inflammation.

5. Conclusion

Our present study revealed that the knockdown of LSD1 meliorated Ox-LDL-stimulated NLRP3 activation and inflammation through promoting autophagy via SESN2-mediated PI3K/Akt/mTOR pathway. This may provide a novel direction for exploring the therapeutic strategy of atherosclerosis.

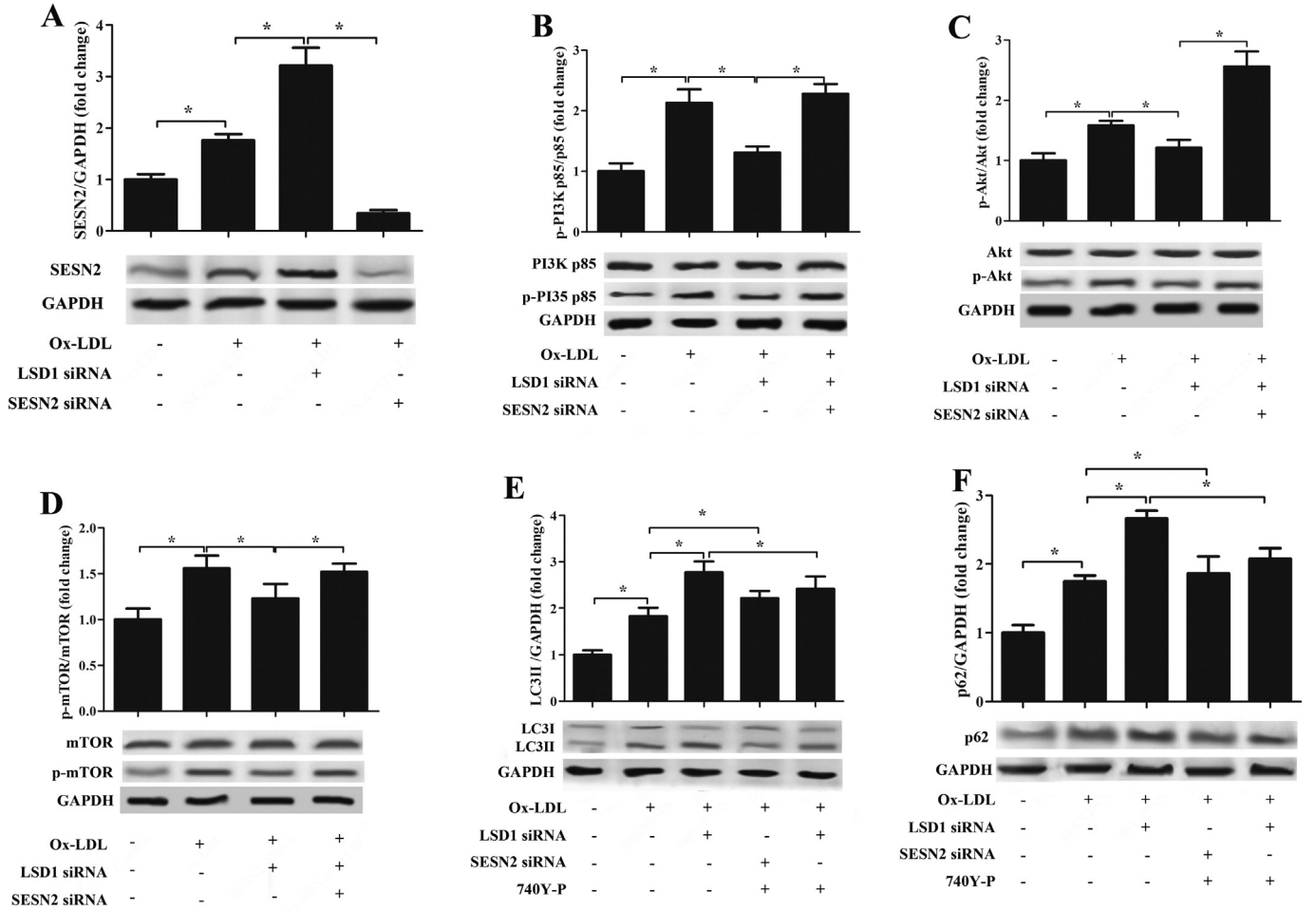

Fig. 5. Interfered LSD1 depresses the activation of PI3K/Akt/mTOR pathway. Following the 24 h transfection of 50 nM LSD1 siRNA and/or SESN2 siRNA, RAW264.7 cells were stimulated by ox-LDL for 24 h at the concentration of 50 mg/L. (A) Determination was conducted using western blotting for the expression of SESN2 in cells transfected with LSD1 siRNA or SESN2 siRNA and treated with ox-LDL or non-ox-LDL. PI3K/Akt/mTOR pathway, and the quantified results were expressed in the next three figures. (B) Western blot was implemented to detect the related protein expressions of p-PI3K. (C) Western blot assay showed the alteration of p-Akt expression. (D) The effect of siRNAs on mTOR activation in ox-LDL-stimulated cells was detected by western blot assay. (E and F) A group of RAW264.7 cells was transfected with 50 nM LSD1 siRNA and/or treated with 20 nM 740Y-P, followed by 24 h stimulation of 50 mg/L ox-LDL. Western blot method was used to detect the protein expression level of LC3II (E) and p62 (F). Every experiment was repeated three times with six replicates for each group, and mean ± SEM was the final manifestation of the result. Statistical analysis was performed by one-way ANOVA, $^{*}P < 0.05$ between groups of comparisons.

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Author contribution

Tao Chen and Xiaozhen Zhuo conceived and designed the study. Xiaozhen Zhuo, Yan Wu, Yanjie Yang, Li Gao and Xiangrui Qiao performed the experiments. Xiaozhen Zhuowrote the paper. Tao Chen reviewed and edited the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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