Original Research Article

Lysine demethylase 1A exacerbates LPS-induced inflammation of vascular smooth muscle cells through modulation of NF-κB activation

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Abstract

Purpose: To study the effect of lysine demethylase 1A (LSD1) on inflammatory responses of vascular smooth muscle cells (VSMCs), and investigate the mechanism.

Methods: VSMCs were treated with lipopolysaccharide (LPS). Overexpression and knockdown of LSD1 in VSMCs were performed by transfecting with LSD1 overexpression plasmid and small interfering RNAs (siRNAs), respectively. Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) were used to measure protein and mRNA levels. Enzyme-linked immunosorbent (ELISA) assay was used to determine the levels of inflammatory cytokines.

Results: Phosphorylation of LSD1 (p-LSD1) was significantly increased in LPS-induced VSMCs. Monocyte chemoattractant protein-1 and IL-6 levels were also increased by LPS, but attenuated by LSD1 knockdown in VSMCs. Activation of NF-κB was increased by LPS, but was also decreased by LSD1 knockdown. Level of methylated p65 (p65-me) in VSMCs was increased by treatment with SET7/9 (p65 methyltransferase), but this effect was attenuated by overexpression of LSD1. Besides, the increased levels of MCP-1 and IL-6 induced by overexpression of LSD1 were reversed by NF-κB signaling inhibitor, PDTC.

Conclusion: LSD1 exacerbates LPS-induced inflammation of VSMCs through NF-κB activation via p65 demethylation, which indicates that LSD1 might be a potential target for the treatment of cardiovascular diseases.

Keywords: Vascular smooth muscle cells, Lysine demethylase 1A, Phosphorylation, NF-κB, p65, Demethylation

INTRODUCTION

Vascular smooth muscle cells (VSMCs) are main components of the vascular wall, contributing primarily to maintain the stability of blood flow and pressure [1,2]. Abnormal proliferation and inflammatory response of VSMCs may result in various vascular diseases such as vascular

stenosis and atherosclerosis after vessel transplant [3,4]. During inflammation induced by lipopolysaccharide (LPS) or other factors, VSMCs produce extensive inflammatory cytokines related to vascular lesions, acceleration of atherosclerosis, and formation of susceptible plaques [5,6]. Therefore, it is of great importance to investigate the mechanism underlying excessive inflammation in VSMCs for the diagnosis and treatment of cardiovascular diseases.

Lysine demethylase 1A (LSD1), also known as lysine (K)-specific demethylase 1A (KDM1A), could demethylate histone H3 [7]and is involved in the regulation of inflammation. In hepatitis B virus-associated glomerulonephritis, LSD1 level was notably increased, and promoted the release of proinflammatory mediators and Toll-Like Receptor 4 [8]. Therefore, LSD1 is considered a regulator of renal inflammation [8].

In a previous study, protein kinase Cα (PKCα)-LSD1-nuclear factor-κB (NF-κB) signaling was a critical for LPS-induced inflammatory response in mice and bone marrow-derived macrophages (Raw264.7 cells) [9]. During inflammatory response, increased LSD1 phosphorylation is induced by PKCα, and LSD1 phosphorylation accelerated the demethylation of p65 and increased the stability of p65 [9]. The inflammatory injury was alleviated after inhibition of LSD1 or PKCα activity [9]. Therefore, LSD1 might be a novel target for regulating the inflammatory response by NF-κB signaling. However, it is unclear whether LSD1 affects the inflammatory response of VSMCs.

Therefore, this study is intended to investigate the effect of LSD1 on excessive inflammatory responses of VSMCs and reveal the underlying regulatory mechanism of this process.

EXPERIMENTAL

Cell culture

VSMCs were maintained in a CO_2 incubator (5 %; 37 °C) using Dulbecco's Modified Eagle's Medium (DMEM) with fetal bovine serum (10 %, FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml; Gibco, Grand Island, NY, USA). To establish an inflammatory model of VSMCs, VSMCs were treated with LPS (10, 100, or 1000 ng/ml) for, 30, 60 or 120 min. In indicated experiments, VSMCs were also treated with 80 µmol/l ammonium pyrrolidinedithiocarbamate (PDTC; NF-κB inhibitor).

RNA extraction and quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from VSMCs using RNeasy Mini Kit (Qiagen, Valencia, CA) and measured by s NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA). Complementary DNA (cDNA) was performed by reverse transcription and qPCR was performed with the following sequences of primers (Table 1). GAPDH was used as control.

Western blot

Total protein was extracted from VSMCs using radioimmunoprecipitation assay and the concentration of the extracted protein was quantified. Forty micrograms of extracted protein was subjected to 12 % SDS-PAGE and transferred to a PVDF membrane, which was then blocked by 4 % non-fat milk for 1 h. The PVDF membranes were incubated with primary antibodies for 12 h and secondary antibody (anti-rabbit IgG antibody) for 1 h. The primary antibodies were anti-LSD1 antibody (1:500, Abcam), anti-p-LSD1 antibody (1:500, Abcam), anti-p65 antibody (1:500, Abcam), and anti-lamin A/C antibody (1:500, Abcam). The membranes were visualized using ECL Plus reagents, and the bands were quantified.

Enzyme-linked immunosorbent assay (ELISA)

The cell supernatants of VSMCs were collected and MCP-1 and interleukin (IL)-6 levels were detected using ELISA kits (Abcam, Cambridge, MA).

Table 1: Sequence of the primers for PCR

Gene name	Forward	Reverse
LSD1	5'-GAGGGAATCCTATGGCTGTGG-3'	5'-AAACTCTTGCTCTACCATTTCATCT-3'
MCP-1	5'-GCACGTTTCAGTGAGCATCG-3'	5'-CAGCAGAGTGGGTGGATTCT-3'
IL-6	5'-TACCCCCAGGAGAAGATTCCA-3'	5'-CCTCTTTGCTGCTTTCACACA-3'
GAPDH	5'-TGAGAACGGGAAGCTTGTCA-3'	5'-GCATCGCCCCACTTGATTTT-3'

Cell transfection

Small interfering RNA (siRNA) (siNC), siRNA for LSD1 (siLSD1), and LSD1 overexpression plasmid were synthesized at Shanghai GenePharma Co., Ltd. For cell transfection, 4 × 10^5 VSMCs were seeded into each well of a 6-well plate and cultured in DMEM for 24 h. The VSMCs were then transfected with siNC, siLSD1, or LSD1 overexpression plasmid using Lipofectamine 3000 reagent (ThermoFisher Scientific, Waltham, MA). VSMCs were collected after 48 h of transfection.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (Chicago, IL, USA) and GraphPad Prism 7 (La Jolla, CA, USA). All data were shown as mean ± standard deviation (SD). Comparison between two groups or among multiple groups was made using Student's t-test or one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. Each experiment was repeated at least three times.

RESULTS

LPS-induced LSD1 phosphorylation in VSMCS

To investigate the role of LSD1 in the LPS-induced inflammatory response of VSMCs, the phosphorylation level of LSD1 (p- LSD1) in VSMCs that treated with LPS was determined. As shown in Figure1A, western blot results showed no significant differences in total LSD1 levels between VSMCs treated with LPS and that without LPS treatment. The protein level ofp-LSD1 was significantly higher in VSMCs treated with 100 or 1000 ng/mL LPS, while p-LSD1 protein expression was not affected by 10 ng/mL LPS as compared to that without LPS treatment. Therefore, the ratio of p-LSD1/LSD1 was significantly higher in the 100 and 1000 ng/mL LPS-treated groups than in the control group ($p < 0.01$).

The protein expression of p-LSD1 was examined in VSMCs that treated with 1000 ng/mL LPS for 30, 60, and 120 min. As shown in Figure 1B, western blot results showed no significant differences in total LSD1 levels among various groups. However, levels of p-LSD1 were significantly increased in VSMCs treated with 1000 ng/mL LPS for 60 and 120 min when compared to that without LPS treatment. Hence, the ratio of p-LSD1/LSD1 was significantly higher in groups that treated with 60 and 120 min than that in the control group ($p < 0.01$).

These results indicated that high concentrations of LPS (100 and 1000 ng/mL) and long treatment time significantly increased p-LSD1 protein expression.
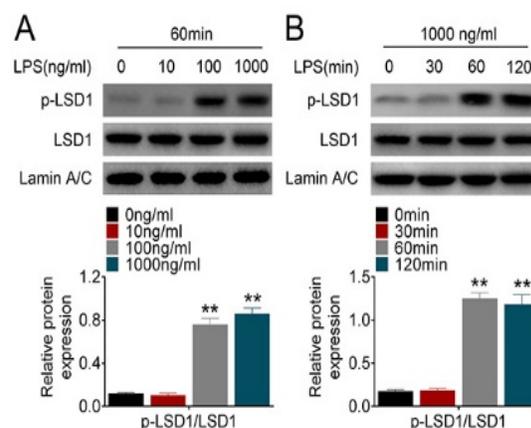

Figure 1: Effect of LPS on LSD1 phosphorylation in VSMCs. VSMCs were treated with 10, 100, or 1000 ng/ml LPS for 60 min (A) and 1000 ng/ml LPS for 30, 60, or 120 min (B), and were assessed by western blotting; **$p < 0.01$ compared to control group

Knockdown of LSD1 attenuated LPS-induced inflammation and inhibited NF-KB activation in VSMCS

The levels of inflammatory factors MCP-1, IL-6 and p65 were assessed in LPS-induced VSMCs after knockdown of LSD1.Results from qRT-PCR and western blot showed that the mRNA and protein levels of LSD1were significantly reduced in VSMCs that transfected with siLSD1-1 or siLSD1-2 when compared to that transfected with siNC ($p < 0.05$, Figure 2 A and B). The efficiency of siLSD1-2 was higher than that of siLSD1-1, which was thus used in subsequent experiments.

Next, the levels of inflammatory factors MCP-1 and IL-6 were determined in LPS-induced VSMCs transfected with siLSD1. Results from qRT-PCR and ELISA assay showed that levels of MCP-1 and IL-6 mRNA and protein were significantly higher in LPS-induced VSMCs when compared to the non–LPS-treated group ($p < 0.01$) and that MCP-1 and IL-6 levels were decreased after VSMCs were transfected with siLSD1 ($p < 0.01$, Figure 2 C and D).

Results also showed that the level of p65 was significantly increased in VSMCs induced by LPS compared to the control group, while its level was significantly decreased when transfected with siLSD1 (all $p < 0.01$, Figure 2 E). In addition, the level of p-LSD1 in LPS-induced VSMCs was increased compared to the control group, while its level was decreased in LPS-induced VSMCs

after transfected with siLSD1. Therefore, the ratio of p-LSD1/LSD1 was significantly higher in LPS-induced VSMCs, which was significantly after transfected with siLSD1 (*p* < 0.01. These results indicated that knockdown of LSD1 attenuated LPS-induced inflammation in VSMCs and inhibited NF-κB activation.
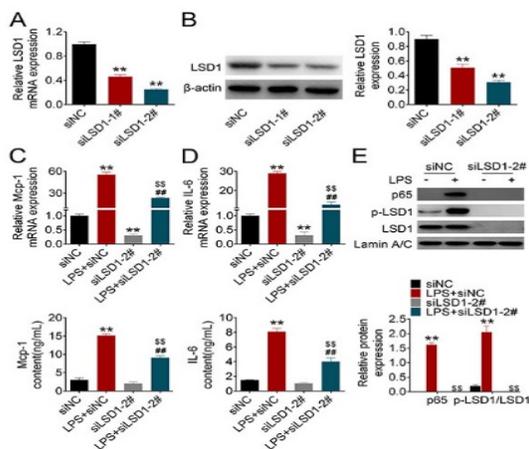

Figure 2: Effect of LSD1 knockdown on the expression of MCP-1, IL-6 and p65in LPS-treated VSMCs. VSMCs were transfected with two kinds of siLSD1, and the mRNA (A) and protein (B) levels of LSD1 were determined using qRT-PCR and Western blot. The levels of MCP-1 (C) and IL-6 (D) were determined using qRT-PCR and ELISA, and the levels of p65, LSD1, and p-LSD1 were quantified using Western blot (E). ***p < 0.01 compared to siNC group; **##**p* < 0.01 compared to the siLSD1 group; **$$**p* < 0.01 compared to LPS + siNC group

Knockdown of LSD1 inhibited NF-KB activation via p65 demethylation

To p65 levels were examined in VSMCs that transfected with siLSD1 and treated with LPS for different time periods. The protein level of p65 in VSMCs treated with LPS for 30, 60, and 120 min were significantly increased when compared to that without LPS treatment (*p* < 0.01, Figure 3 A). However, the p65 levels in VSMCs that treated with in LPS (30, 60, and 120 min) and transfected with si-LSD1 were significantly decreased when compared to that transfected with si-NC and treated with LPS (*p* < 0.01, Figure 3 A).The levels ofp-LSD1 were also increased in LPS-treated groups 30, 60, and 120 min), while this upregulation was reversed by knockdown of LSD1 (*p* < 0.01). Therefore, the ratio of p-LSD1/LSD1 was significantly increased in LPS-treated VSMCs (30, 60, and 120 min), but this effect was significantly decreased by knockdown of LSD1 (*p* < 0.01, Figure 3 A).

For further analysis, VSMCs were transfected with LSD1 overexpression plasmid. Results from

qRT-PCR showed that the expression of LSD1 was significantly increased in VSMCs transfected with LSD1 overexpression plasmid when compared to control group (*p* < 0.01, Figure 3 B). The VSMCs were then treated with LPS (1000 ng/mL) and SET7/9, a p65 methyltransferase, to determine whether p65 methylation was involved in the regulatory effects of LSD1 on NF-κB activation. Western blot results showed that methylated p65 (p65-me) levels were clearly increased in VSMCs treated with SET7/9 as compared to control group and that this phenomenon was abolished by overexpression of LSD1 (Figure 3 C).

Conversely, p65 level was clearly decreased in VSMCs treated with SET7/9 when compared to the control group, whereas p65 levels were increased in VSMCs that co-treated with SET7/9 and LSD1 plasmid compared to that treated with SET7/9 alone. The ratio of p65-me/p65 was significantly higher in VSMCs treated with SET7/9, and this effect was abolished by LSD1 overexpression (*p* < 0.01). In addition, the protein levels of p-LSD1 and LSD1 were increased in LPS-induced VSMCs co-treated with SET7/9 and LSD1 plasmid compared to that treated with SET7/9 alone. The ratio of p-LSD1/LSD1 showed no significant differences among four groups. These results indicated that knockdown of LSD1 inhibited NF-κB activation through p65 demethylation.
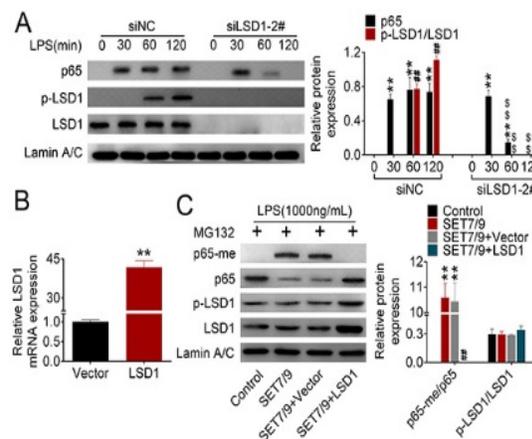

Figure 3: Knockdown of LSD1 inhibited NF-κB activation through p65 demethylation. (A) VSMCs were treated with 1000 ng/ml LPS for 0, 30, 60, or 120 min and transfected with siLSD1, and the protein levels of p65, p-LSD1, and LSD1 were determined using Western blot. (B) VSMCs were transfected with LSD1 overexpression plasmid, and the mRNA levels of LSD1 were measured using qRT-PCR. (C) VSMCs were transfected with LSD1 overexpression plasmid and treated with SET7/9, and levels of p65-me, p65, LSD1, and p-LSD1were determined using Western

blot; **$p < 0.01$ compared to control group; ##$p < 0.01$ compared to SRT7/9 + vector group

LSD1 exacerbated VSMC inflammation by activation of NF-κB

To study whether LSD1 could regulate inflammation in VSMCs by NF-κB activation, LPS-treated VSMCs were treated with PDTC, an inhibitor of NF-κB. Results showed that p65 levels were significantly increased by LSD1 overexpression, which was reversed by PDTC ($p < 0.05$, Figure 4 A). In addition, qRT-PCR and ELISA results showed that the levels of MCP-1 and IL-6 were also significantly increased by LSD1 overexpression when compared to the control group ($p < 0.01$, Figure 4 B and C). After treated with PDTC, the mRNA and protein levels of MCP-1 ($p < 0.05$) and IL-6 ($p < 0.01$) significantly decreased (Figure 4 B and C). These results indicated that LSD1 exacerbated inflammation in VSMCs by activation of NF-κB.
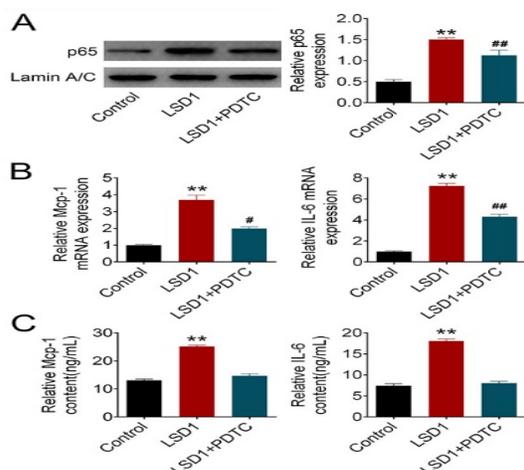

Figure 4: LSD1 exacerbated the inflammatory response in VSMCs by activation of NF-κB. (A) The protein level of p65 in VSMCs transfected with LSD1 overexpression plasmid and treated with PDTC was determined by Western blot. Levels of MCP-1 and IL-6 in VSMCs transfected with LSD1 overexpression plasmid and treated with PDTC were determined by qRT-PCR (B) and ELISA (C); **$p < 0.01$, compared to control group; #$p < 0.05$, compared to LSD1 group; ##$p < 0.01$, compared to LSD1 group

DISCUSSION

Excessive inflammatory response of VSMCs is a contributing factor to various vascular diseases [3,10]. Therefore, it is critical to elucidate the regulatory mechanism of excessive inflammation in VSMCs, which may contribute to investigate novel targets for cardiovascular diseases therapy. In some studies, LSD1 was shown to be involved in the regulation of inflammatory

response, suggesting that LSD1 might regulate the inflammatory response of VSMCs.

Increasing studies have reported that activation of p-LSD1 contributes considerably to many diseases. For example, Boulding *et al* [11] found that the activation of p-LSD1was critical for epithelial-to-mesenchymal transition activity and chemotherapy resistance of breast cancer cells. In addition, p-LSD1 expression level induced by PKCα is involved in circadian rhythmicity [12]. This study firstly showed that the p-LSD1 level was increased in VSMCs that treated with LPS. Consistently, Kim *et al* [9] found that p-LSD1 was activated by PKCα in the inflammatory response. Therefore, p-LSD1 was activated during the inflammatory process of VSMCs.

To further study the role of LSD1 in the inflammatory response of VSMCs, the effects of LSD1 on the levels of inflammatory factors MCP-1 and IL-6 and activation of NF-κB were determined. Knockdown of LSD1 decreased the levels of MCP-1, IL-6, and p65. These results are different from studies from Janzer *et al* [13] and Liu *et al* [14]. Janzer *et al.* found that downregulation of LSD1 increased inflammatory response in cancer cells via demethylation of lysine histone H3 [13]. Liu *et al.* also found that loss of LSD1 activity induced the production of pro-inflammatory cytokines and that this phenomenon may be related to demethylation of lysine histone H3 [14]. These differences may be caused by different regulatory mechanisms. The NF-κB signaling pathway is considered to be a prototypical proinflammatory pathway [15]. The results in our study showed that p65 levels were decreased by LSD1 knockdown, and the ratio of p65-me/p65 was increased after SET7/9 treatment, and these effects were abolished by overexpression of LSD1. These findings suggested that knockdown of LSD1 caused the inhibition of NF-κB activation via demethylation of p65. These results are consistent with results of Kim *et al* [9], revealing thatp-LSD1 could cause p65 demethylation.

In addition, lysine-specific demethylase 2A (KDM2A) also repressed NF-κB activity through p65 demethylation at K218 and K221 methylation sites[16]. LSD1, also known as KDM1A, along with KDM2A, are from the same family of molecules. It seems that LSD1 may also regulate p65 demethylation at K218 and K221 methylation sites, and thereby regulate NF-κB activation.

Finally, this study studied whether LSD1 could affect the inflammatory response by the regulation of NF-κB. The results showed that MCP-1 and IL-6 levels were increased by LSD1

overexpression, and this effect was reversed by PDTC, an inhibitor of NF-κB activation. Accumulating evidences showed that NF-κB activation promoted inflammatory responses [17,18]. In general, LSD1 phosphorylation was activated during inflammatory response of VSMCs, and LSD1 promoted the activation of NF-κB via demethylation of p65, which then exacerbated the inflammatory response.

Excessive inflammation of VSMCs may be a critical component of cardiovascular disease. A mechanism by which LSD1 contributes to excessive inflammation of VSMCs was defined in this study. Thus, LSD1 may be a potential target for the treatment of cardiovascular diseases.

CONCLUSION

Lysine demethylase 1A exacerbates LPS-induced inflammation of VSMCs through modulation of NF-κB activation via p65 demethylation. Thus, LSD1 may be a potential target for the treatment of cardiovascular diseases.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. ZQQ and WHL conceived and designed the experiments, LL analyzed and interpreted the results of the experiments, MLS performed the experiments.

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