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Simvastatin attenuates the lipopolysaccharide-induced inflammatory response of rat pulmonary microvascular endothelial cells by down-regulating toll-like receptor 4 expression

Research Article

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Abstract: Objective: The therapeutic potential of simvastatin as an anti-inflammatory agent was explored by investigating its effect on the lipopolysaccharide (LPS)-induced inflammatory response in rat pulmonary microvascular endothelial cells (RPMVECs). Methods: RPMVECs were isolated and the mRNA and protein levels of different toll-like receptors (TLR) were assessed by qRT-PCR and western blotting. The LPS-induced expressions of TLR4, TNF-α and iNOS were analyzed in RPMVECs treated with different concentrations of simvastatin for different times. NF-κB activation was examined by immuofluroscence, luciferase reporter assay and western blotting. Results: TLR4 is abundantly expressed in RPMVECs, and its expression is induced by LPS stimulation. Simvastatin inhibited LPS-induced TLR4 expression at the mRNA and protein levels in a time-dependent manner (p<0.01), and alleviated inflammation in RPMVECs by inhibiting the release of inflammatory factors such as TNF-α and iNOS. Further study indicated that simvastatin significantly attenuated NF-κB activity by inhibiting the degradation of IκB-α. Pretreatment with pyrrolidine dithiocarbamate (PDTC) and knock-down of TLR4 expression by RNA interference down-regulated the LPS-induced inflammatory response in RPMVECs. Conclusion: Simvastatin inhibits the LPS-induced inflammatory response in RPMVECs by down-regulating TLR4 expression, suggesting its role as a potential inhibitor of LPS-induced inflammation

Keywords: Simvastatin • Pulmonary microvascular endothelial cells • TLR4 • LPS • Mechanism

1. Introduction

Pulmonary trauma, severe infection, and hemorrhage can result in acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), which are common pulmonary diseases in humans, especially in compromised patients undergoing surgery [1]. Studies have confirmed that lipopolysaccharide (LPS) plays important roles in the development and progression of ALI or ARDS. Increased serum endotoxin or LPS of Gram-negative bac-

teria can cause pulmonary inflammation leading to ALI and predicts multiple organ failure (MOF), particularly during sepsis [2]. LPS recognition by the host receptor is a critical step leading to the activation of signal transduction cascades in a variety of cells in the lung [3]. Toll-like receptor 4 (TLR4) is required for the innate immune response to LPS of Gram-negative bacteria [4]. Studies have shown that mice with TLR4 gene deletion (TLR4-/-) (C57BL/10ScCr) and mice with a natural point mutation (C3H/HeJ) in the TLR4 gene are unresponsive to system-

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ic LPS. Although the role of TLR4 in pulmonary inflammation is still debatable, studies have shown that LPS rather than TLR4 is directly involved in the induction of pulmonary immune responses [5]. Furthermore, LPS-induced CD14-dependent and -independent (CD11b-dependent) signaling pathways in the lung are entirely dependent on TLR4 and blocking TLR4 might be beneficial for lung injury caused by LPS from Gram-negative pathogens [6].

Simvastatin, which lowers cholesterol by inhibiting 3-hydroxyl-3-methylglutaryl (HMG)-CoA reductase, has been widely prescribed to hyperlipidemic patients at risk for cardiovascular disease [7]. Clinical trials have demonstrated that simvastatin therapy significantly reduces cardiovascular events. In addition to its cholesterol-lowering properties, simvastatin has pleiotropic effects such as the improvement of endothelial function, stabilization of atherosclerotic plaques, the control of thrombosis, and the reduction of oxidative stress [8,9]. The effect of simvastatin on inflammation, which plays a central role in cardiovascular disease, has been studied extensively. The documented anti-inflammatory effects of simvastatin include inhibition of reactive oxygen species formation; decreased expression of adhesion molecules and proinflammatory cytokines such as TNF-α, IL-1β, IL-6, CD40 ligand, growth factors and chemokines; decreased T cell activation; and NO synthesis [10-12].

Although the anti-inflammatory properties of simvastatin have been described, its effect on LPS-induced inflammatory gene expression has not been well-established, and conflicting observations have been reported. Therefore, more studies are necessary to define the role of simvastatin in LPS-stimulated inflammation. In the present study, we show that simvastatin inhibits the LPS-induced inflammatory response in rat pulmonary microvascular endothelial cells (RPMVECs) by down-regulating TLR4 expression, suggesting that simvastatin is a potential inhibitor of LPS-induced inflammation.

2. Materials and methods

2.1 Materials

Heparin, thapsigargin (TG), and ionomycin were purchased from Calbiochem. FBS was from Intergen. Rat tail collagen IV and ECs growth supplement were obtained from Collaborative Biomedical Products. Chambered glass coverslips were from Lab Tek. Microspheres (50 μm) were obtained from NEN. RPMI 1640, serum-free Aim V medium, gentamicin, and viokase were purchased from GIBCO-BRL. Rat epidermal growth factor was from Biomedical Technologies. DMEM was obtained from Cellgro Mediatech. Fura 2-AM and pluronic acid were from Molecular Probes. HEPES was from Re-search Organics. Hyaluronidase I, Krebs-Henseleit buffer, FITC-dextran (average mol wt 12,000, 72,000, and 150,000), bovine serum albumin (BSA), and all other analytical reagents were purchased from Sigma.

2.2 Culture and isolation of RPMVECs

RPMVECs were isolated and cultured in our laboratory using a modification of the method of Ryan et al. [13]. Briefly, rat lungs (male Sprague-Dawley, 350−400g) were initially perfused without recirculation with 300 mL of Krebs-Henseleit buffer containing 8% BSA. The perfusate was changed to serum-free Aim V medium containing 8% BSA, 50 mg of hyaluronidase I, and collagen IV-coated 50-μm microspheres in a volume of 100 mL. The direction of perfusate flow alternated from anterograde to retrograde (0.03 mL•min^{-1}•g body wt^{-1}), and perfusate containing the EC-bound microbeads was collected on ice. The microbeads with cells were washed three times with RPMI supplemented with 25% FBS and resuspended in RPMI containing 20% rat serum, 0.1% gentamicin, and EC-conditioned medium (2:1). Pure primary cultures were established and cells were grown in 20% FBS-DMEM containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, 25 μg/mL fungizone, and 10 μg/mL gentamicin until passage 10 and with 10% FBS thereafter. The cell stocks were harvested by scraping without proteases for passage until used for experimentation. Cells were studied between passage 4 and passage 20.

2.3 Western blot analysis

Proteins extracted from RPMVECs were analyzed by immunoblotting as described elsewhere [14]. Briefly, near confluent cells were collected as low-speed pellets and homogenized in TM-PI buffer supplemented with EDTA (0.5 mM) and NaF (10 mM). Fifty micrograms of total protein from the supernatant obtained after centrifugation at 100,000 × g was precipitated using methanol-chloroform, re-dissolved in loading buffer, and subjected to 10% polyacrylamide gel electrophoresis followed by electrotransfer to a nitrocellulose membrane (Schleicher & Schuell, Inc.). After 1 h pre-incubation with 1% blocking buffer (Boehringer Mannheim) at room temperature, immunoblotting was performed with different antibodies as described elsewhere [14]. Membranes were incubated in POD conjugated secondary IgG antibodies and the immunoreactivity was visualized using the BM Teton POD substrate (Boehringer Mannheim) and 1.5% hydrogen peroxide. Monoclonal mouse anti-human TLR4 (1:200; Serotec, UK) and monoclonal rabbit anti-human I-κB (1:1000; ABCAM, UK) antibodies were used to detected the expression of TLR4 and I-κB, respectively. GAPDH (1:5000; Chemicon, USA) was used as an internal control.

2.4 RNA extraction, RT-PCR and qRT-PCR

The mRNA expression of TLRs, TNF-α and INOS in RPMVECs was detected by qRT-PCR as described elsewhere [14]. Amplification and detection were performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) starting with 1μl cDNA and SYBR Green Realtime PCR Master Mix (Toyobo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers are including in Table 1. The qRT-PCR conditions were as follows: 10 min at 94°C, 40 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 30 s, and extension at 72°C for 60 s. The relative expression of targets was analyzed by the comparative cycle threshold (Ct) method, according to the equation: 2-ΔCt [ΔCt = Ct (target gene) - Ct (GAPDH)]. RT-PCR reactions were carried out on 1 μg of cDNA. PCR products were electrophoresed in agarose and stained with ethidium bromide. The density of bands was determined using 1.58 Image software over a range of cycles, and a linear relationship between cycle number and product density was detected. The primers used are listed in Table 1. All experiments were performed in triplicate.

2.5 Immunofluorescence staining

Cells were incubated on six-well plates overnight at 37°C. Slides were fixed in 4% paraformaldehyde for 30 min. and immersed in 1% Triton X-100 for 10 min at room temperature. Cells were incubated in rabbit polyclonal anti-Factor VIII Related Antigen (1:100, Santa Cruz biotechnology®) and goat anti-p65 (1:500, Santa Cruz biotechnology®) primary antibodies overnight at 4°C, and in fluorescein isothiocyanate (FITC)-coupled secondary antibodies (Santa Cruz biotechnology®) for 1 h at room temperature. Slides were stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min before examination under a fluorescence microscope.

2.6 RNA interference

Briefly, RPMVECs were grown to 60−70% confluence and transfected with signal silence-negative siCon (ConsiRNA) or siRNA targeting TLR4 (siTLR4, Shanghai Genomeditech, Shanghai, China) at a concentration of 50 nM using Oligofectamine Reagent (Shanghai Genomeditech, Shanghai, China) for 48 h, according to the manufacturer's instructions. The sequences for siRNA targeting were as follows: TLR4 sense, 5′-CUUUAUC-CAACCAGGUGCATT-3′ and TLR4 antisense, 5′- UG-CACCUGGUUGGAUAAAGTT-3′.

2.7 TNF-α and iNOS assays

ELISAs were adapted to measure mouse TNF-α and iNOS (R&D Systems, Chantilly, VA, USA) in bronchoalveolar lavage fluid (BALF) and performed according to the manufacturers' protocols.

2.8 Statistical analysis

SPSS16.0 software was used for the statistical analysis. Data were obtained from three independent experiments with duplicate determinations and expressed as means ± SE (standard error). Statistical analysis was performed by one-way ANOVA and comparisons were made using Fisher's multiple comparison test. P< 0.01 was considered significant.

3. Results

3.1 Identification of RPMVECs

As shown in Figure 1A and B, RPMVECs were spindle-shaped or polygonal, uniform in size, and arranged in a cobblestone mosaic-like pattern at 12 h. Cells became elongated during growth and showed a characteristic capillary-like structure. To further characterize the cells as RPMVECs, the expression of factor VIII-related antigen, a specific marker of endothelial cells, was determined by immunofluorescence staining, which showed that the vast

Table 1. The primers used in this study.

| Gene	Upstream primer	Downstream primer	length (bp)	Tm
TNF-α	5-ttcggggtgatcggtcccaaca-3	5-catagtcggggggcagccttgtccct-3	296	59
iNOS	5-caccatcagaggggatcctgcca-3	5-gctggggcagtctccattgaca-3	976	60
GAPDH	5-cgagaaatatgacaactccctca-3	5-cagccccagcatcaaaggtgga-3	487	58
TLR1	5-agtacagggatacaggccgtca-3	5-gatccatttgcccggaacaa-3	725	60
TLR2	5-cgttccgcagaaccaactctgt-3	5-ggattccgtcatgggccaattca-3	730	60
TLR4	5-ctgaaaatgt gccttcaaaa ca-3	5-aggtctaagtacttcaggttgt-3	487	57
TLR5	5-aactgcagctgcctctaaggact-3	5-AGTGAgagcctatggccagaga-3	250	62
TLR6	5-gattggtgagtctgaagtacct-3	5-acccttcaggaaattaacggga-3	550	60
TLR7	5-tgcacctgag ctgtgttctt ct-3	5-ttgtttctatgtgatggagggt-3	278	58
TLR9	5-cctttcagaacctgacccgtct-3	5-gagctcactgaacgatttcgaat-3	651	60
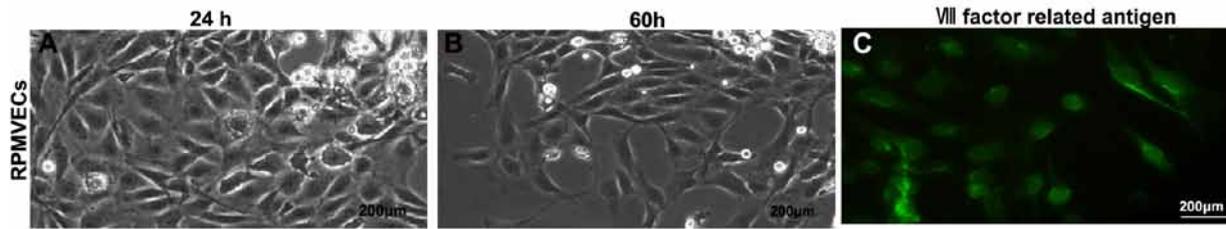
Figure 1. Identification of RPMVECs.
A: The shape of RPMVECs at 24 hours in ordinary microscope; B: The shape of RPMVECs at 60 hours in ordinary microscope;
C: The immunofluorescence staining of the VII factor related antigen in RPMVECs.

majority of cells expressed factor VIII-related antigen in the cytoplasm (Figure 1C). Approximately 95% of RPMVECs were positive for factor VIII-related antigen expression.

3.2 Expression of toll-like receptors in RPMVECs

The expression of various TLR family members in RPM-VECs was determined by RT-PCR. RPMVECs showed positive expression of TLR1, TLR2, TLR4, TLR6, and TLR7 whereas TLR5 and TLR9 were not detected. Quantification of the expression of TLRs showed that TLR4 was expressed at significantly higher levels (9.9) than TLR1, TLR2, TLR6, and TLR7 (p<0.01) (Table 1, Figure 2A and B).

3.3 Simvastatin suppresses LPS-induced TLR4 expression

As LPS induces TLR4 expression in vascular cells, we treated RPMVECs with LPS and showed its effect on the induction of TLR4 expression (Figure 3A and B).

We then examined the effect of simvastatin on LPS-induced TLR4 expression in RPMVECs. Simvastatin treatment for 24 and 48 h inhibited TLR4 mRNA and protein expression in a time-dependent manner, with a significant inhibitory effect at 48 h (p <0.01) (Table 3, Figure 3C and D).

Table 3. The influence of simvastatin on the TLR4 protein expression in different time.

LPS	+	+	+	+
SIM	0	24h	48h	72h
TLR4	100.0±1.60	95.8±2.10*	76.7±3.40**	33.3±4.23***

*vs 0h P<0.01, ** vs 24h P<0.01, *** vs 48h P<0.01.*

3.4 Simvastatin suppresses the LPS-induced production of the inflammatory cytokines TNF-a and iNOS

LPS induces the production of an array of pro-inflammatory mediators, including cytokines such as TNF-α

Table 2. The mRNA expression of TLRs in the rat pulmonary microvascular endothelial cells.

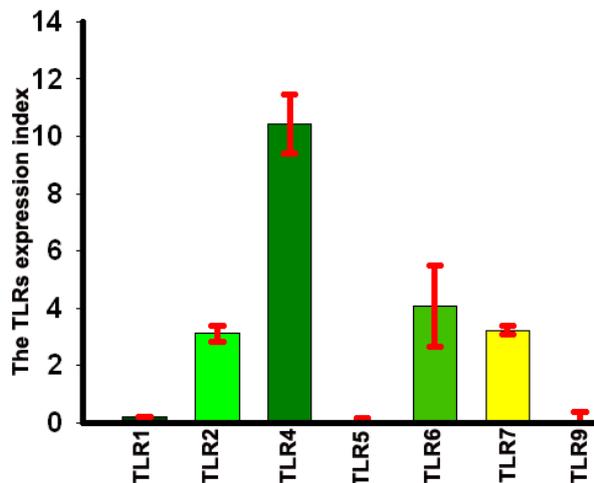| TLR1	TLR2	TLR4	TLR5	TLR6	TLR7	TLR9
0.21±8.64e-3	3.12±0.29	10.43±1.04**	0.10±0.09	4.07±1.42	3.24±0 .14	0.11±0.30

*mean±SD ** P<0.01.*

Figure 2. TLRs expression in RPMVECs. The expression of a variety of TLR family members in RPMVECs by qRT-PCR.
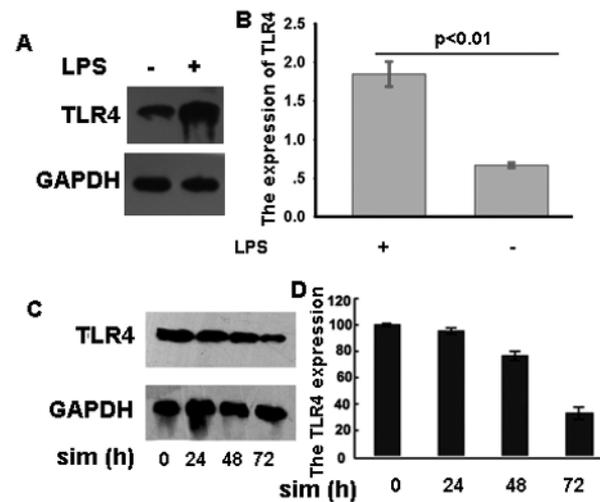

Figure 3. Simvastatin suppresses the LPS-induced TLR4 expression.
A and B: The LPS induce TLR4 expression in RPMVECs; C and D: the simvastatin inhibit the TLR4 mRNA and protein expression.

and inducible nitric oxide synthase (iNOS) [15]. Therefore, we examined the effect of simvastatin on the LPS-induced production of TNF-α and iNOS in RPMVECs. Our results showed that simvastatin dose-dependently decreased the production of TNF-α and iNOS induced by LPS in RPMVECs (Figure 4).

3.5 Simvastatin suppresses the LPS-induced inflammatory response by inhibiting NF-κB transcriptional activity

Increasing evidence supports that the LPS-induced inflammatory response is NF-κB dependent [16,17]. Therefore, we examined the effect of simvastatin on LPS-induced NF-κB activation. Inhibition of NF-κB signaling may occur by a variety of mechanisms, such as the enhanced expression of IκB-α, which forms an inactive cytoplasmic complex with the p65-p50 heterodimeric complex. The protein levels of IκB-α were determined by western blot analysis using specific anti-IκB-α and anti-phosphorylated IκB-α antibodies. Treatment of LPS-stimulated RPMVECs with simvastatin at different concentrations resulted in a related increase in the level of IκB-α and the reduction of phosphorylated IκB-α (Figure 5A). Because activation of NF-κB requires IκB-α degradation, we assessed the effect of simvastatin on the level of phosphorylated IκB-α. IκB-α levels were reduced in RPMVECs, but this reduction was not observed when cells were co-incubated with a high concentration of simvastatin. Furthermore, immunofluores-
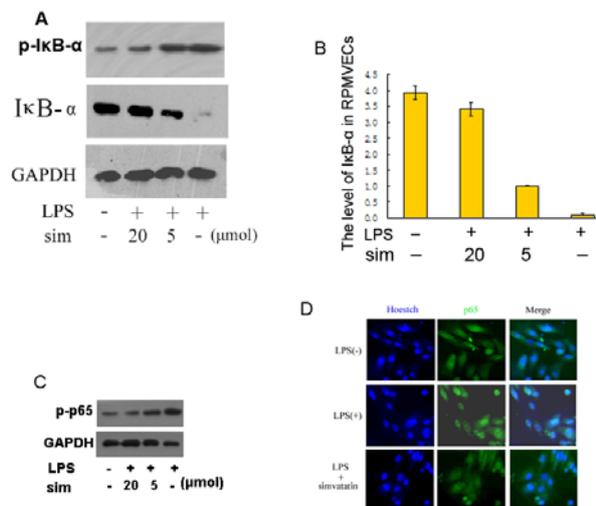
cence staining showed that LPS-treated RPMVECs accumulated p65 in the nucleus, which was not observed in cells co-incubated with simvastatin and LPS (Table 4, Figure 5A, B and D), confirming the western blot results. Pretreatment of cells with pyrrolidine dithiocarbamate (PDTC), a class of antioxidants reported to be potent inhibitors of NF-κB, and TLR4 interference also inhibited the LPS-induced expression of TNF-α and iNOS in RPMVECs (Figure 5C and Table 5).

Table 4. The effect of simvastatin with the different concentration on the IκB-α.

LPS	-	+	+	+
SIM	-	20 (μmol/L)	5 (μmol/L)	-
IκB-α	3.9±0.22	3.4±0.20*	1.0±0.01**	0.1±0.03***

*** LPS- / SIM- vs LPS alone, P<0.01; ** LPS- / SIM- vs. LPS+/SIM 5 μmol/L P<0.01; * LPS- / SIM- vs. LPS+/SIM 20μmol/L P<0.05.

Table 5. The PDTC and siTLR4 inhibit the LPS-induced TNF-α and iNOS expression.

	Control	PDTC group	siTLR4 gourp
LPS	+	+	+
iNOS	9.75 ± 2.21	3.16 ± 0.97	2.9 ± 0.88
TNF-α	10.48 ± 3.05	4.11 ± 1.10	3.86 ±1.23

Note: the iNOS and TNF-α were tested by ELISA, the concentration of simvastatin is 20 μmol.

4. Discussion

LPS is a major recognition marker for the detection of Gram-negative bacteria by the host and a powerful initiator of the inflammatory response to infection [18]. Activation of cells by LPS is mediated by TLR4, a member of the highly conserved family of TLR proteins, which are specialized in the recognition of microbial components [15]. Here, we show that simvastatin attenuates the LPS-induced inflammatory response of RPMVECs by down-regulating TLR4 expression. To the best of our knowledge, the present study is the first to show that simvastatin blocks LPS-induced inflammation in RPMVECs by downregulating TLR4 gene expression. Furthermore, we showed that simvastatin significantly reduced the inflammatory activation of RPMVECs by inhibiting LPS-induced NF-κB activity.

LPS stimulates the synthesis and release of proinflammatory cytokines such as TNF-α, IL-1β, IL-8 and IL-6 from monocytes and macrophages [19,20]. These cytokines can further activate monocytes, neutrophils and lymphocytes, causing cellular injury and tissue damage. Among inflammatory cells, activated alveolar macrophages and infiltrated/activated neutrophils play a major role in airway mucus hypersecretion by releasing various kinds of inflammatory cytokines and proteinases [21]. TNF-α has been shown to induce the expression and
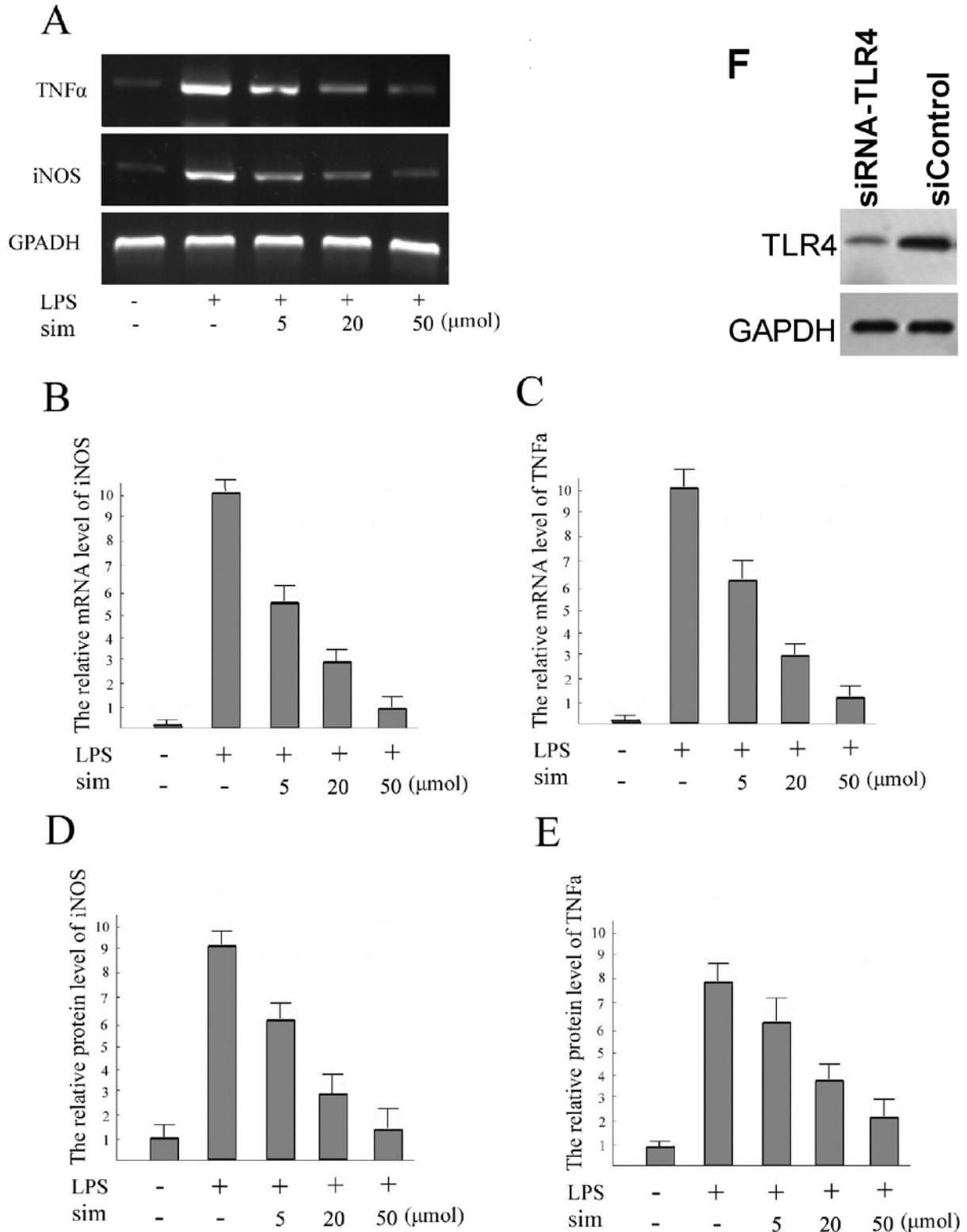

Figure 5. Simvastatin suppresses the LPS-induced inflammatory by inhibiting NF-κB transcriptional activity.
A and B: Western blot showed that the simvastatin increase the level of IκB-α in RPMVECs exposed to LPS; C: Western blot showed that the simvastatin decrease the phosphorylation level of P65; D: the RPMVECs treated by LPS with the accumulation of p65 in nucleus by immunofluorescence staining, while not in cells co-incubated with simvastatin and LPS.

Figure 4. Simvastatin suppresses the LPS-induced inflammatory cytokines TNF-a and iNOS.
A : Simvastatin suppresses the LPS- inducing the mRNA expression of TNF-α and iNOS in RPMVECs; B: Simvastatin suppresses the
LPS-inducing the mRNA expression of iNOS in RPMVECs; C: Simvastatin suppresses the LPS-inducing the mRNA expression of TNF-α
in RPMVECs; D: Simvastatin suppresses the LPS-inducing the protein expression of iNOS in RPMVECs; E: Simvastatin suppresses the
LPS -inducing the protein expression of TNF-α in RPMVECs.; F: The interference of TLR4 by siRNA in RPMVECs.

production of respiratory mucins [22]. IL-8 plays an important role in neutrophil elastase-induced goblet cell metaplasia and Muc5ac production in the airway epithelium [23]. Statins attenuated the infiltration of inflammatory cells (macrophages, neutrophils and eosinophils) in allergic asthma and in an acute lung injury model, and altered the release of proinflammatory cytokines such as TNF-α and IL-8 in vitro and in vivo [8]. In the present study, simvastatin attenuated LPS-induced TLR4 expression and the release of the inflammatory cytokines TNF-α and IL-8 in LPS-stimulated RPMVECs. Moreover, the release of inflammatory cytokines was markedly decreased by TLR4 interference. These data suggest that simvastatin inhibits the inflammatory response of RPMVECs by downregulating TLR4 expression.

Simvastatin belongs to a class of cholesterol-lowering statins that are widely used to reduce cardiovascular morbidity and mortality in patients with or without coronary artery disease [10]. Simvastatin inhibits acute and chronic inflammatory responses in a cholesterol-independent manner by interfering with endothelial adhesion and leukocyte migration to sites of inflammation [24]. In rats with normal blood cholesterol levels, simvastatin ameliorated immunopathology in an acute TNBS colitis model by blocking neutrophil accumulation in the small intestine and lowering serum TNF-α levels [25]. Although the anti-inflammatory potential of statins has been demonstrated in several studies, the mechanisms underlying the regulation of airway inflammation by statins has not been fully elucidated [26]. Several possible mechanisms of action may explain the effect of statins on the reduction of inflammation including the modulation of cholesterol content and the prevention of the prenylation of signaling molecules and the subsequent down-regulation of gene expression. Statin-

sensitive signaling molecules include Rho guanosine triphosphatases (GTPases), MAPK and Akt. RhoA and p38 MAPK signaling pathways are important mediators of LPS-induced proinflammatory cytokine production (e.g. TNF-α and IL-8) and TNF-α-induced inflammatory damage. Moreover, RhoA acts upstream of MAPK in an LPS-activated pathway [27]. RhoA expression and p38 phosphorylation levels were shown to be positively correlated with the concentration of the inflammatory cytokines TNF-α and IL-8 in BALF, and the expression of Muc5ac mRNA in the lung [28]. Here, we showed that the LPS-induced inflammatory response of RPMVECs is mediated by the NF-κB signaling pathway, and simvastatin attenuates the LPS-induced activation of NF-κB signaling, thereby decreasing inflammatory cytokine production and inflammation in RPMVECs.

In conclusion, our results indicate that simvastatin inhibits the LPS-induced inflammatory response of RPMVECs by downregulating TLR4 expression at the mRNA and protein levels. The reduction of the inflammatory response of RPMVECs by simvastatin is mediated by inhibition of NF-κB signaling.

Acknowledgments

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