



TLR4/NF- κ B signaling pathway-mediated and oxLDL-induced up-regulation of LOX-1, MCP-1, and VCAM-1 expressions in human umbilical vein endothelial cells

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ABSTRACT. This study aimed to investigate the function and signaling pathway of Toll-like receptor 4 (TLR4) in oxidized low-density lipoprotein (oxLDL)-induced up-regulated expressions of oxidized LDL receptor 1 (LOX-1), monocyte chemoattractant protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with different oxLDL concentrations (0, 20, 40, 60, and 80 μ g/mL) for 24 and 48 h. The influence of oxLDL on TLR4, LOX-1, MCP-

1, and VCAM-1 expressions in HUVECs was detected by real-time polymerase chain reaction and Western blot analysis. HUVECs were transfected with small interfering RNA targeting TLR4 (siTLR4), in which protein expressions of LOX-1, MCP-1, and VCAM-1, and the nuclear translocation of NF- κ B (P50) were detected by Western blot. After 48 h of processing HUVECs with pyrrolidine dithiocarbamate (PDTC), protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1 were detected by Western blot. OxLDL induced a concentration-dependent up-regulation of mRNA and protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1 in HUVECs ($P < 0.001$). siTLR4 significantly reduced protein expressions of LOX-1, MCP-1, VCAM-1, and reduced the NF- κ B (P50) nuclear translocation ($P < 0.001$). PDTC significantly inhibited protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1 ($P < 0.001$). Results of this study demonstrate that the TLR4/NF- κ B signaling pathway has an important function in the up-regulation of oxLDL-induced expressions of LOX-1, MCP-1, and VCAM-1 in HUVECs.

Key words: Oxidized low-density lipoprotein (LDL); NF- κ B; Toll-like receptor-4; Lectin-like oxidized LDL receptor-1; Vascular cell adhesion molecule-1; Monocyte chemotactic protein-1

INTRODUCTION

The oxidized low-density lipoprotein (oxLDL) is one of the most important risk factors for atherosclerosis (Zeibig et al., 2011; Tew et al., 2012). One of the earliest effects of atherosclerosis is endothelial cell dysfunction and inflammatory chemokine secretion to increase the number of mononuclear macrophages (Simionescu, 2007). Two main oxLDL receptors can be found on the surface of endothelial cells: lectin-like oxidized LDL receptor 1 (LOX-1) and Toll-like receptor 4 (TLR4) (Nagase et al., 1997; Moriwaki et al., 1998; Edfeldt et al., 2002; Fisker et al., 2008; Sivapalaratnam et al., 2011). TLR4 is a type I transmembrane protein. In-depth studies related to immune and inflammatory mechanisms of atherosclerosis have revealed that TLR4, as a kind of pattern recognition receptor in innate immunity, has a crucial function in the genesis and development of atherosclerosis that is related to induced up-regulation of chemokines, adhesion molecules, and other inflammatory factors (Miller et al., 2005; Geng et al., 2010). LOX-1 is a type II membrane surface glycoprotein, which belongs to the C-type lectin family and is mainly expressed in endothelial cells. LOX-1 supports the specific binding, internalization, and degradation of oxLDL. A previous study demonstrated that polymorphonuclear leukocytes adhered to LOX-1-coated slides, which could be inhibited by anti-LOX-1 antibodies. Thus, LOX-1 was considered to be a member of the adhesion molecule family (Li and Mehta, 2000). The induction and specific mechanism of TLR4 in the up-regulation of expressions of LOX-1 and other inflammatory factors, such as the monocyte chemoattractant protein 1 (MCP-1) and the vascular cell adhesion molecule 1 (VCAM-1), in endothelial cells, and the influence of oxLDL have not yet been reported. This paper presents results of a preliminary study of this process.

MATERIAL AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC-12) were cultured adherently in a 1640 medium containing 10% fetal bovine serum. Cells in the logarithmic phase were used for experiments. At 12 h before cells reached the logarithmic phase, the medium was replaced with a fresh serum-free medium for cell synchronization in the G0 phase.

Influence of oxLDL on TLR4, LOX-1, MCP-1, and VCAM-1 expressions

Experiments were divided into five groups: 1) HUVECs were incubated with 0 $\mu\text{g/mL}$ oxLDL + 1640 medium; 2) HUVECs were incubated with 20 $\mu\text{g/mL}$ oxLDL + 1640 medium; 3) HUVECs were incubated with 40 $\mu\text{g/mL}$ oxLDL + 1640 medium; 4) HUVECs were incubated with 60 $\mu\text{g/mL}$ oxLDL + 1640 medium; and 5) HUVECs were incubated with 80 $\mu\text{g/mL}$ oxLDL + 1640 medium. RNA or protein was detected after a culture time of 24 or 48 h.

TLR4 small interfering RNA (siRNA) transfection

Sequences of three pairs of TLR4 siRNAs (Guangzhou RiboBio Co., Ltd., Guangzhou, China) are shown in Table 1. HUVECs were transfected only after cells on the cell culture plate reached 50% confluence. First, 10 μL TLR4 siRNA was diluted with 475 μL serum-free 1640 medium, which were mixed by blending. Approximately 15 μL HiPerFect transfection reagent (QIAGEN GmbH, Hilden, Germany) was then added to the solution. The solution was left to stand for 10 min to form the transfection complex. This solution was gently added on a 6-well plate containing 1500 μL serum-free 1640 medium until a final TLR4 siRNA concentration of 100 nM was obtained. Cells were then cultured for 6 h in the incubator. The transfection of the fluorescent-labeled siRNA into the cells was detected using an inverted fluorescence microscope. In this study, siRNA was fluorescently labeled as Cy3-siRNA. Transfection was deemed to be successful when maroon fluorescence was detected in the cytoplasm after excitation with green fluorescence. The change in mRNA expression was detected after 24 h of successful transfection, and the change in protein expression was detected after 48 h.

Table 1. The sequence of TLR4 siRNA.

	Sense strand (5'-3')	Antisense strand (3'-5')
siTLR4_001	CCAACCAGGUGCAUUUAAA dTdT	dTdT GGUUGGUCCACGUAAAAUUU
siTLR4_002	GGACAACCAGCCUAAAGUA dTdT	dTdT CCUGUUGGUCGGAUUUCAU
siTLR4_003	CUACUACCUCGAUGAUUU dTdT	dTdT GAUGAUGGAGCUACUAUAA

Grouping

Based on the influence of TLR4 siRNA on the up-regulation of oxLDL-induced protein expressions of MCP-1, VCAM-1, and LOX-1 in HUVECs, experiments were divided into

four groups: 1) HUVECs were incubated with 1640 medium for 48 h; 2) HUVECs were incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h; 3) HUVECs were incubated with 100 nM siTLR4_001 + HiPerFect transfection reagent + 1640 medium for 6 h, and then incubated further with 80 μ g/mL oxLDL + 1640 medium for 42 h; and 4) HUVECs were incubated with 100 nM negative control siRNA + HiPerFect transfection reagent + 1640 medium for 6 h, and then incubated further with 80 μ g/mL oxLDL + 1640 medium for 42 h.

Based on the different concentrations of pyrrolidine dithiocarbamate (PDTC), the inhibitor of NF- κ B, experiments were divided into three groups: 1) HUVECs were incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h; 2) HUVECs were pretreated with 50 μ M PDTC for 1 h and incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h; 3) HUVECs were pretreated with 100 μ M PDTC for 1 h and incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h.

Based on the influence of TLR4 siRNA on the nuclear translocation of NF- κ B (P50), experiments were divided into four groups: 1) HUVECs were incubated with 1640 medium for 48 h; 2) HUVECs were incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h; 3) HUVECs were incubated with 100 nM siTLR4_001 + HiPerFect transfection reagent + 1640 medium for 6 h, and then incubated further with 80 μ g/mL oxLDL + 1640 medium for 42 h; 4) HUVECs were incubated with 100 nM negative control siRNA + HiPerFect transfection reagent + 1640 medium for 6 h, and then incubated further with 80 μ g/mL oxLDL + 1640 medium for 42 h.

Based on the influence of PDTC on the up-regulation of oxLDL-induced protein expressions of MCP-1, VCAM-1, LOX-1, and TLR4 in HUVECs, experiments were divided into two groups: 1) HUVECs were incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h; 2) HUVECs were pretreated with 100 μ M PDTC for 1 h and incubated with 80 μ g/mL oxLDL + 1640 medium for 48 h.

RT-PCR

Total RNA was extracted, of which 2 μ L was used to synthesize the cDNA by reverse transcription. Approximately 1 μ L reverse transcription product was used for PCR cycle amplification. The primer sequences were as follows: the upstream primer of LOX-1 was 5'-CTGCCAGCCTGAAGTCCATT-3' and the downstream primer was 5'-TCTGTCTGTCTGTCCGTAAGTG-3', with an amplified fragment of 340 bp. The upstream primer of MCP-1 was 5'-CTCGCTCAGCCAGATGCAAT-3' and the downstream primer was 5'-TGTCTGGGGAAAGCTAGGGG-3', with an amplified fragment of 299 bp. The upstream primer of TLR4 was 5'-TGGTGGAAGTTGAACGAATGG-3' and the downstream primer was 5'-CAGCAAGAAGCATCAGGTGAA-3', with an amplified fragment of 186 bp. The upstream primer of VCAM-1 was 5'-TATCTGCATCGGGCCTCACT-3' and the downstream primer was 5'-AGGAAAAGAGCCTGTGGTGC-3', with an amplified fragment of 277 bp. The upstream primer of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was 5'-CAAGGTCATCCATGACAACCTTG-3' and the downstream primer was 5'-GTCCACCACCCTGTTGCTGTAG-3', with an amplified fragment of 496 bp. The PCR was performed in the following conditions: 5 min at 94°C for pre-denaturation, 45 s at 9°C for denaturation, 45 s at 60°C for annealing, 1 min at 72°C for elongation with a total of 35 circulations, followed by another 2 min at 72°C for elongation.

After PCR, the RT-PCR product was subject to 1.5% agarose gel electrophoresis. Approximately 5 μ L GAPDH and 10 μ L other products were loaded, respectively, with ethidium bromide staining. Electrophoresis bands were photographed using the UVP gel image analysis system to analyze the gray value of each target and GAPDH gene. The ratio of the gray values represented the expression of the target gene.

Protein extraction from adherent cells

Cells to be treated were placed on ice, the medium was then removed, and the cells were washed thrice with phosphate-buffered saline at 4°C. The cell culture bottle was inverted on the filter paper to dry the residual liquid as thoroughly as possible. The cell lysate and phenylmethylsulfonyl fluoride (ratio of 94:6) were added into the cell culture bottle and mixed with a pipette to ensure that the lysate was in full contact with the cells. The cell culture bottle was kept flat on the ice and was fully dissociated for 1 min. The liquid was then collected in a 1.5-mL EP tube and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was placed in another set of EP tubes for protein quantitation via the bicinchoninic acid method. A 5X sodium dodecyl sulfate (SDS) gel loading buffer was added with a proportion of 4:1. After blending, EP tubes were carefully inserted into a foam board, which was lightly placed in boiling water for 6 to 8 min to ensure that the proteins were thoroughly denatured. The foam board was then taken out for ice cooling for 10 min, followed by 1 min of centrifugation using a mini-centrifuge, and were stored in a refrigerator at -20°C.

Western blot

A 10% separation gel and a 5% spacer gel were prepared based on instructions provided in the SDS-polyacrylamide gel electrophoresis preparation kit. After the sample was loaded, the separation gel was run at 80 V and the spacer gel was run at 120 V. The proteins were transferred at 200 mA for 2 h and were stained to observe the effect of protein transfer. The film was enclosed with 5% skimmed milk on a shaker for 3 h at room temperature. The film was then incubated overnight with a primary antibody diluted in Tris-buffered saline with Tween (TBST) (1:500) at 4°C. The film was washed three times for 10 min per wash with the TBST on a shaker at room temperature. The film was then incubated with a secondary antibody diluted in TBST (1:1000) for 2 h. The film was then washed three times with TBST on a shaker for 20 min each time. Finally, an ECL chemiluminescence kit was used, and the final X-ray photographic film was obtained after developing and fixing. After scanning, the Quantity One gel image analysis software was used for quantitative analysis. The ratio of gray value of the target protein in each group to that of β -actin or GAPDH represented the expression level of the target protein.

Statistical analysis

All experimental data are reported as means \pm standard deviation, and were analyzed using the Student *t*-test on the GraphPad Prism v5.0 drawing statistical analysis software. $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Influence of oxLDL on TLR4, LOX-1, MCP-1, and VCAM-1 expressions in HUVECs

HUVECs were incubated with different oxLDL concentrations (0, 20, 40, 60, and 80 μ g/mL). After 24 and 48 h, mRNA and protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1 were detected via RT-PCR and Western blot analysis. Results showed that oxLDL induced a concentration-dependent up-regulation of mRNA and protein expressions of the TLR4 (Figure 1), LOX-1 (Figure 2), MCP-1 (Figure 3), and VCAM-1 (Figure 4) genes, and the induction was most significant in the 80 μ g/mL group.

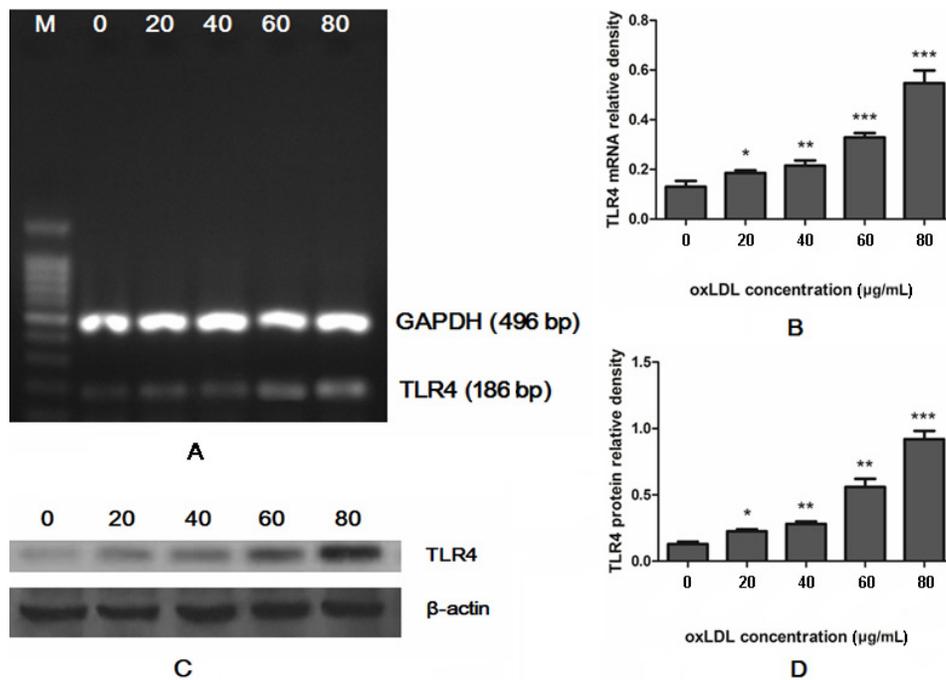


Figure 1. Incubation of HUVECs with oxLDL increased TLR4 mRNA and protein expressions. HUVECs treated with different concentrations of oxLDL (0, 20, 40, 60, 80 μ g/mL), TLR4 mRNA levels detected by RT-PCR (24 h after oxLDL stimulus) (A and B), and protein levels detected by Western blot (48 h after oxLDL stimulus) (C and D). * $P < 0.05$ vs 0 μ g/mL group; ** $P < 0.01$ vs 0 μ g/mL group; *** $P < 0.001$ vs 0 μ g/mL group.

Transfection efficiency of siRNA

The transfection efficiency was observed using an inverted fluorescence microscope, and HiPerFect was used as the transfection reagent. Maroon fluorescence was observed in the majority of HUVECs, which indicated that Cy3-siRNA was successfully transfected into the cells (Figure 5). The most effective siTLR4 pair was screened out for subsequent experiments. Three pairs of siRNA sequences could all induce the down-regulation of TLR4 mRNA expression. The down-regulation was most obvious and intense in siTLR4_001, which was then chosen for subsequent interference experiments (Figure 6).

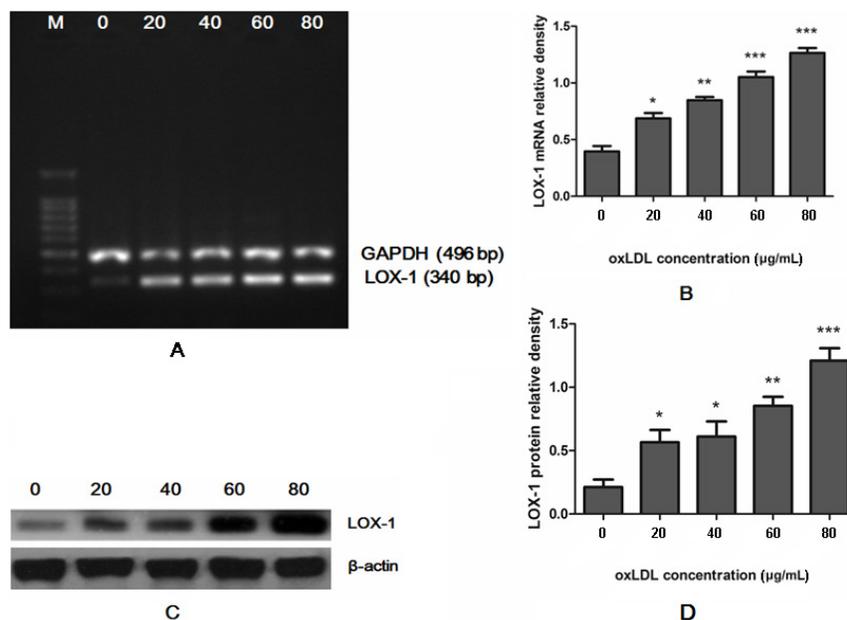


Figure 2. Incubation of HUVECs with oxLDL increased LOX-1 mRNA and protein expressions. HUVECs treated with different concentrations of oxLDL (0, 20, 40, 60, 80 µg/mL), LOX-1 mRNA levels detected by RT-PCR (24 h after oxLDL stimulus) (A and B), and protein levels detected by Western blot (48 h after oxLDL stimulus) (C and D). *P < 0.05 vs 0 µg/mL group; **P < 0.01 vs 0 µg/mL group; ***P < 0.001 vs 0 µg/mL group.

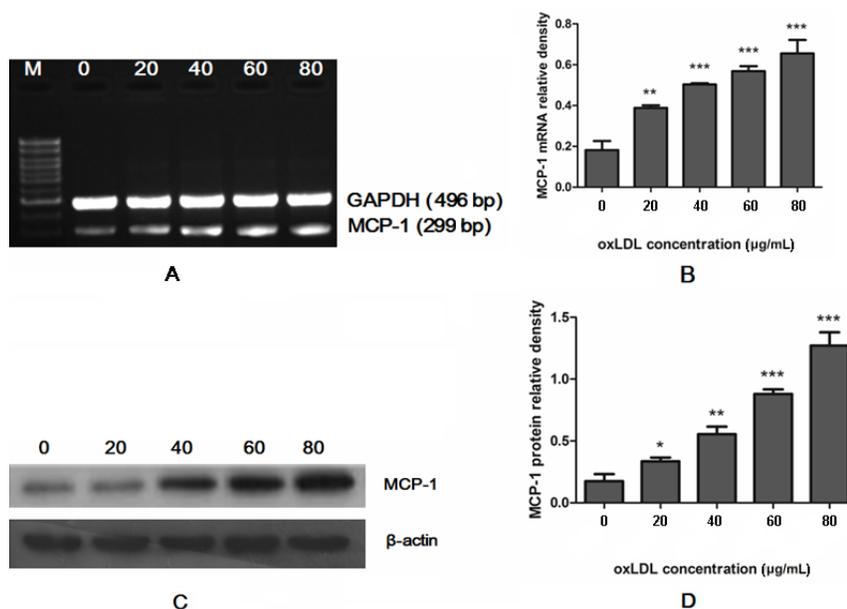


Figure 3. Incubation of HUVECs with oxLDL increased MCP-1 mRNA and protein expressions. HUVECs treated with different concentrations of oxLDL (0, 20, 40, 60, 80 µg/mL), MCP-1 mRNA levels detected by RT-PCR (24 h after oxLDL stimulus) (A and B), and protein levels detected by Western blot (48 h after oxLDL stimulus) (C and D). *P < 0.05 vs 0 µg/mL group; **P < 0.01 vs 0 µg/mL group; ***P < 0.001 vs 0 µg/mL group.

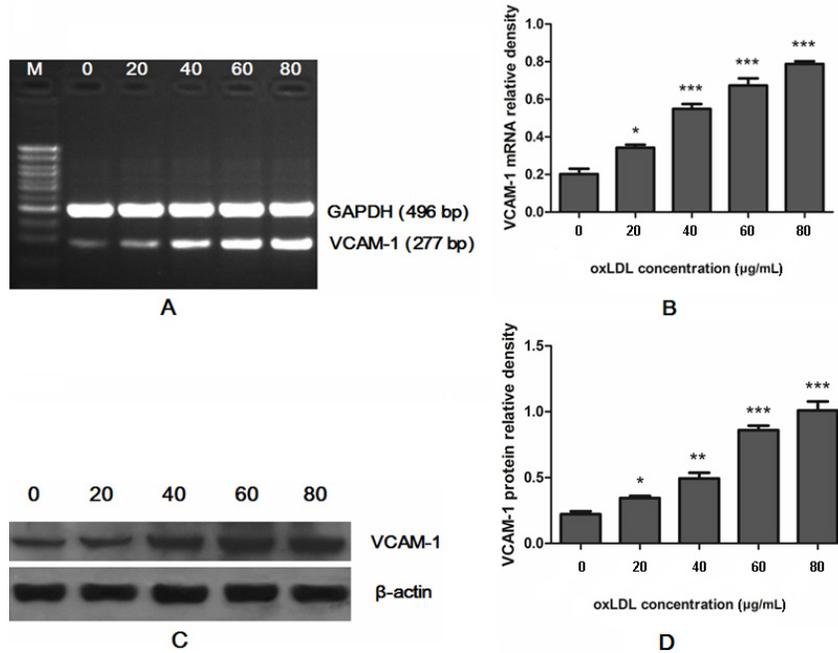


Figure 4. Incubation of HUVECs with oxLDL increased VCAM-1 mRNA and protein expressions. HUVECs treated with different concentrations of oxLDL (0, 20, 40, 60, 80 μ g/mL), VCAM-1 mRNA levels detected by RT-PCR (24 h after oxLDL stimulus) (A and B), and protein levels detected by Western blot (48 h after oxLDL stimulus) (C and D). * $P < 0.05$ vs 0 μ g/mL group; ** $P < 0.01$ vs 0 μ g/mL group; *** $P < 0.001$ vs 0 μ g/mL group.

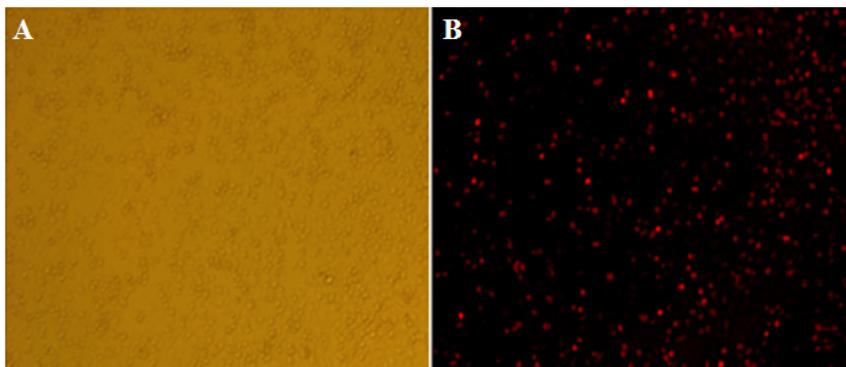


Figure 5. Transfection efficiency of siRNA determined by fluorescence microscopy (10X). A. Light microscope; B. fluorescent microscope.

Influence of TLR4 siRNA on the up-regulation of oxLDL-induced protein expressions of LOX-1, MCP-1, and VCAM-1

Cells were transfected based on the experimental steps, and HiPerFect was used as the transfection reagent. Different reagents were added based on contents contained in the

experimental groups. Changes in protein expressions of LOX-1, MCP-1, and VCAM-1 were detected after 48 h. Figure 7 shows the following observations: compared with group 1, protein expressions of LOX-1, MCP-1, and VCAM-1 significantly increased in the simple oxLDL group ($P < 0.001$); compared with group 2, protein expressions of LOX-1, MCP-1, and VCAM-1 significantly decreased in group 3 with oxLDL and siTLR4 ($P < 0.001$); protein expressions of LOX-1, MCP-1, and the difference in VCAM-1 expression between the negative control group 4 with oxLDL and siRNA and group 2 were not statistically significant. These findings indicated that siTLR4 could inhibit the oxLDL-mediated up-regulation of LOX-1, MCP-1, and VCAM-1, and that the inhibition was not caused by the transfection reagent or other factors. Therefore, TLR4 played a key role in the oxLDL-mediated up-regulation of LOX-1, MCP-1, and VCAM-1 in HUVECs.

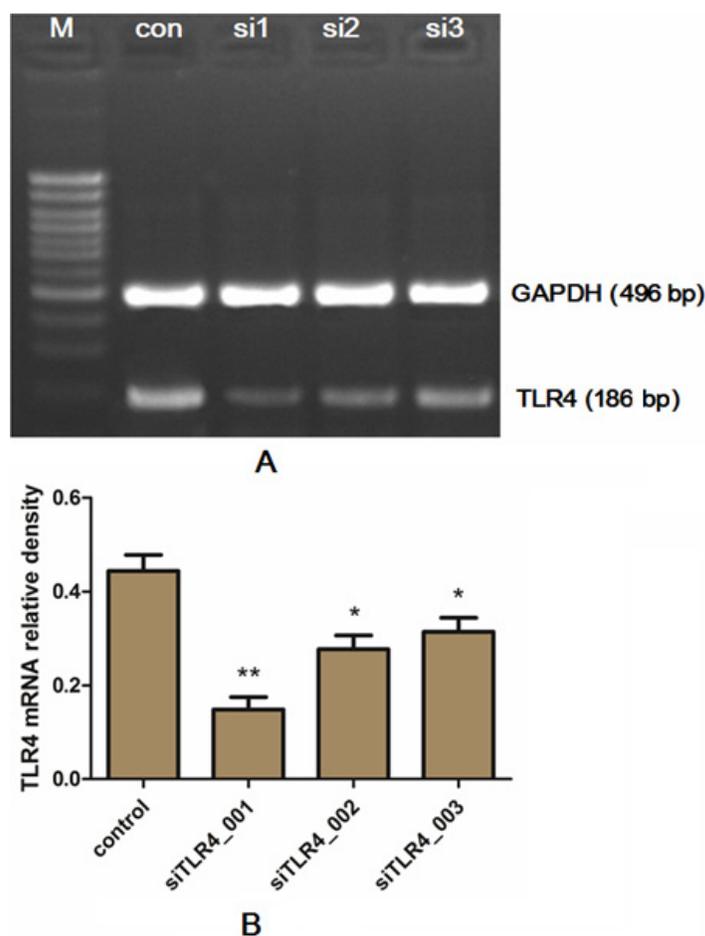


Figure 6. Silencing efficiency of siTLR4. **A.** RT-PCR analysis of TLR4 mRNA levels after consecutively transfecting three pairs of 100 nM distinct siRNA sequences for 24 h. **B.** Semi-quantification of TLR4 mRNA. * $P < 0.05$ vs control group; ** $P < 0.01$ vs control group.

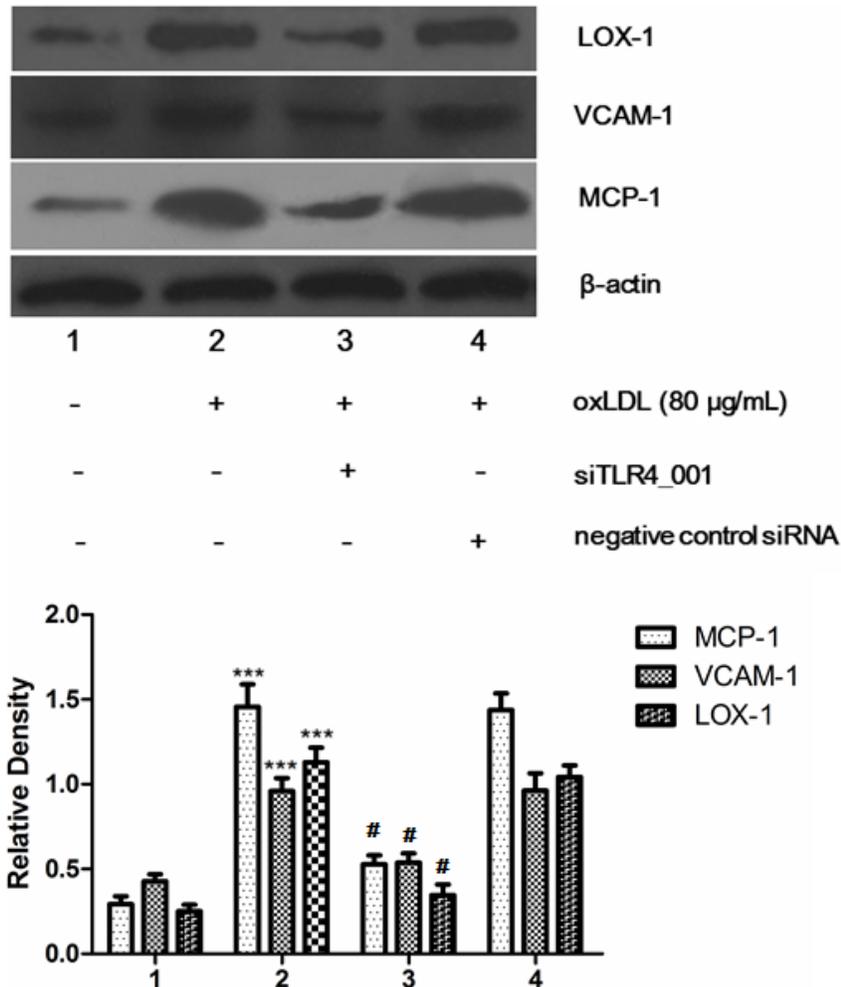


Figure 7. Effect of siTLR4 on the expression of LOX-1, MCP-1, and VCAM-1 induced by oxLDL. HUVECs transfected with siTLR4, then LOX-1, MCP-1, and VCAM-1 protein levels analyzed by Western blot. Columns: 1 = control; 2 = HUVECs treated with 80 μg/mL oxLDL for 48 h; 3 = HUVECs transfected with siTLR4 for 6 h, then treated with 80 μg/mL oxLDL for 42 h; 4 = HUVECs transfected with negative control siRNA for 6 h, then treated with 80 μg/mL oxLDL for 42 h. *** $P < 0.001$ vs group 1; # $P < 0.001$ vs group 2.

Influence of TLR4 siRNA on the nuclear translocation of NF-κB (P50)

Cells were transfected based on the experimental steps, and HiPerFect was used as the transfection reagent. Different reagents were added based on the contents of the experimental groups. NF-κB (P50) protein expression was detected by Western blot after 48 h. In this study, the nuclear protein of NF-κB (P50) was extracted to detect the protein content of active NF-κB (P50) because NF-κB (P50) only exhibits its physiological function in the nucleus. The results showed that oxLDL could significantly induce the up-regulation of the nuclear protein expres-

sion of NF- κ B (P50) by activating the nuclear translocation of NF- κ B, whereas siTLR4 could inhibit the nuclear translocation of NF- κ B (P50) (Figure 8), which indicated that activation of NF- κ B might be partly mediated by TLR4.

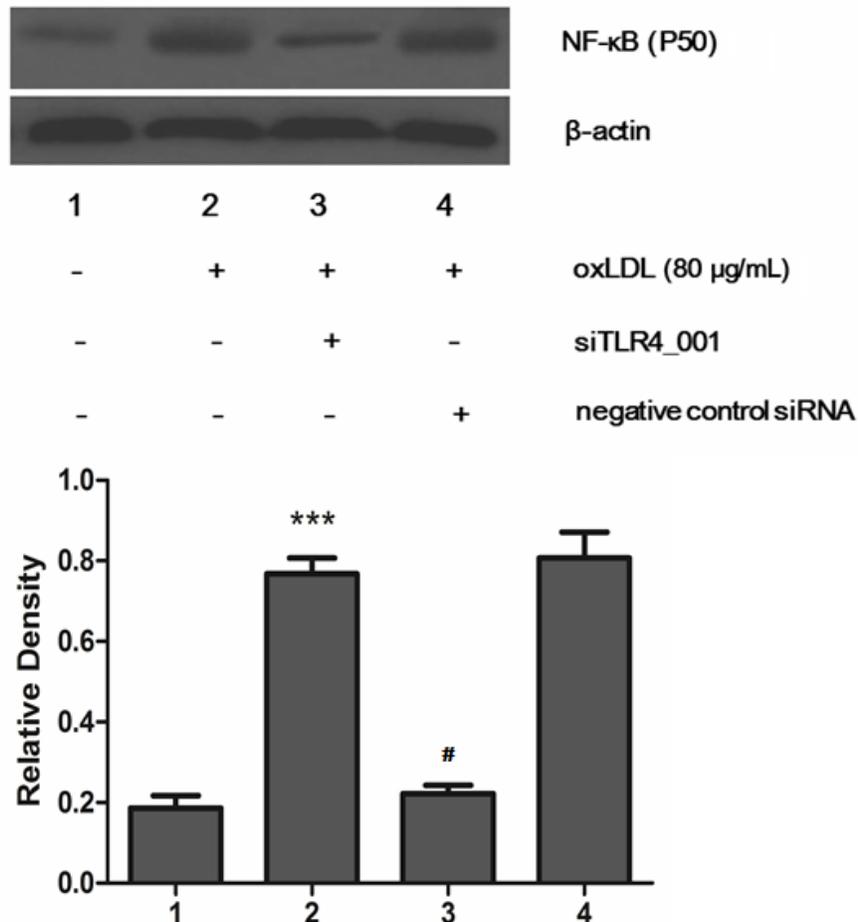


Figure 8. Effect of siTLR4 on the nuclear translocation of NF- κ B (P50) induced by oxLDL. Columns: 1 = control; 2 = HUVECs treated with 80 μ g/mL oxLDL for 48 h; 3 = HUVECs transfected with siTLR4 for 6 h, then treated with 80 μ g/mL oxLDL for 42 h; 4 = HUVECs transfected with negative control siRNA for 6 h, then treated with 80 μ g/mL oxLDL for 42 h. ***P < 0.001 vs group 1; #P < 0.001 vs group 2.

Concentrations of PDTC, the specific inhibitor of NF- κ B

Based on previous reports (Li and Mehta, 2000; Hajishengallis et al., 2008; Chavez-Sanchez et al., 2010), PDTC concentrations of 50 and 100 μ M were selected in the present experiment. The NF- κ B (P50) protein was extracted using the nucleoprotein extraction kit. After synchronization, HUVECs were pretreated with PDTC (0, 50, and 100 μ M) for 1 h. The medium was then replaced with additional oxLDL (80 μ g/mL). The protein content of NF- κ B

(P50) in the nucleus was detected after 48 h for quantization of the nuclear translocation of NF- κ B (P50). Figure 9 shows that 50 and 100 μ M PDTC could both significantly inhibit the nuclear translocation quantity of NF- κ B ($P < 0.001$), especially in 100 μ M PDTC, which was then selected as the treatment concentration in subsequent experiments.

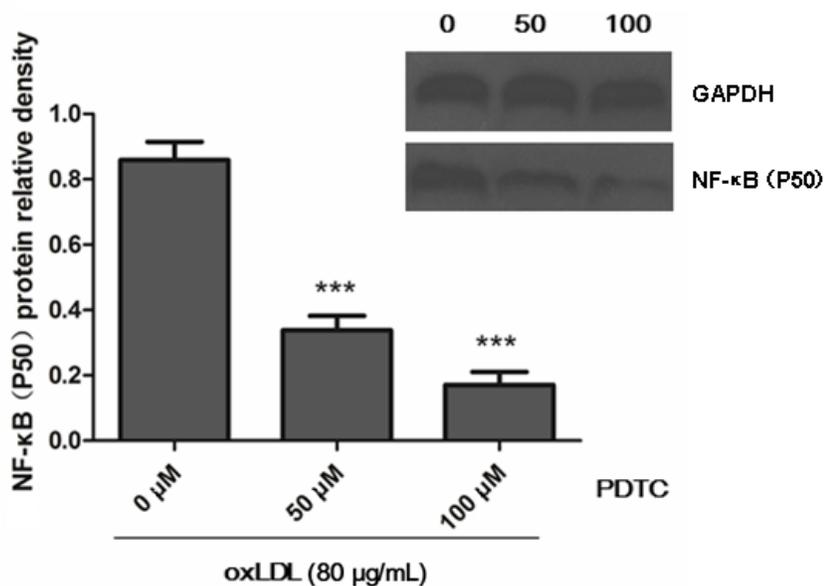


Figure 9. Incubation of HUVECs with PDTC reduces NF- κ B (p50) nuclear translocation. HUVECs pre-incubated with PDTC (0, 50, 100 μ M) for 1 h, and then treated with 80 μ g/mL oxLDL for 48 h. NF- κ B (P50) nuclear translocation detected by Western blot. *** $P < 0.001$ vs 0 μ M group.

Influence of PDTC on the up-regulation of oxLDL-induced protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1

Based on the experimental results described above, 100 μ M was chosen as the PDTC treatment concentration. Figure 10 shows that the protein expressions of TLR4, LOX-1, MCP-1, and VCAM-1 in the PDTC treatment group were all significantly reduced compared with the control group, which indicated that the gene transcriptions of TLR4, LOX-1, MCP-1, and VCAM-1 were regulated by NF- κ B.

DISCUSSION

OxLDL is one of the many risk factors that cause endothelial cell injuries. Chavez-Sanchez et al. (2010) demonstrated that oxLDL could induce the up-regulation of expressions of MCP-1, VCAM-1, intercellular adhesion molecule-1, and other inflammatory factors in endothelial cells to promote inflammation and to increase the adhesion, deposition, and migration of inflammatory cells such as mononuclear cells. In endothelial cells, the two crucial receptors of oxLDL are TLR4 and LOX-1 (Nagase et al., 1997; Moriwaki et al., 1998; Edfeldt

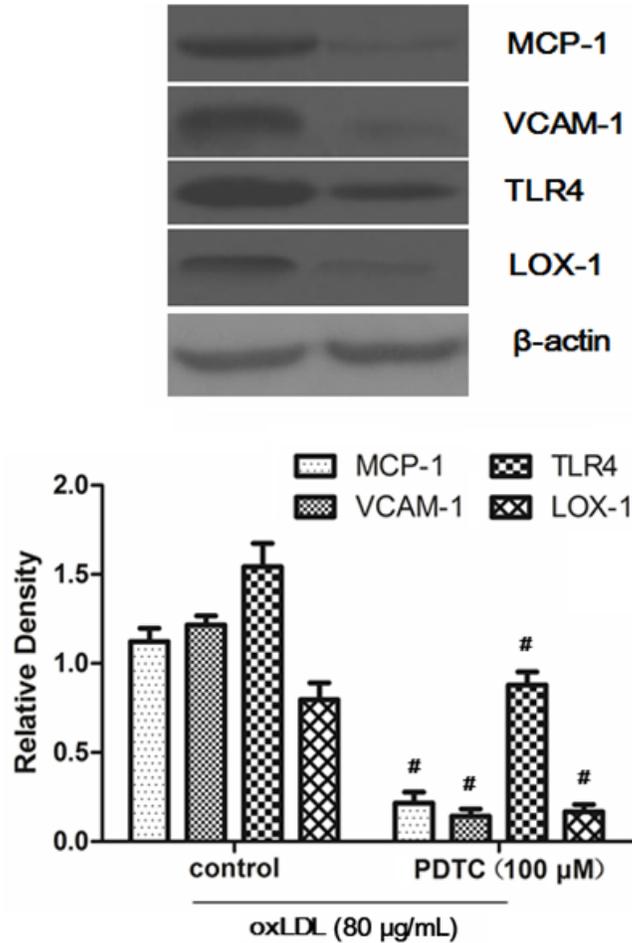


Figure 10. Incubation of HUVECs with 100 μM PDTC reduces MCP-1, VCAM-1, LOX-1, and TLR4 protein expressions. HUVECs pre-incubated with 100 μM PDTC for 1 h, and then treated with 80 μg/mL oxLDL for 48 h. MCP-1, VCAM-1, LOX-1, and TLR4 protein levels detected by Western blot. [#]P < 0.001 vs control group.

et al., 2002; Fisker et al., 2008; Sivapalaratnam et al., 2011). In the present study, oxLDL induced a concentration-dependent up-regulation of TLR4, oxLDL, MCP-1, and VCAM-1 expressions, which is consistent with results of previous studies.

TLRs are important receptors in the innate immune system, and are considered as pattern recognition receptors. TLRs identify both pathogen-associated molecular patterns (LPS, lipopeptid, fungal zymosan, single- or double-stranded RNA, etc.) and autologous damage-associated molecular patterns (oxLDL, C-reactive protein, heat shock protein, hyaluronic acid fragments, and autologous dead cells, etc.) in the body, and activate the immune response. TLR-mediated immunization and inflammation are closely related to atherosclerosis (Hajishengallis et al., 2008). Yang et al. (2011) showed that in atherosclerosis plaque, TLR expression significantly increased, in which TLR2 and TLR4 were the most prominent. Michelsen

et al. (2004) found that after 6 months of a high-cholesterol diet, mice with both TLR4 and apolipoprotein (ApoE) knocked out showed a 55% reduction of plaque area in the aortic sinus compared with the control group. Moreover, compared with the control group, mice with both myeloid differentiation factor 88 (MyD88), which is the downstream gene of TLR4, and ApoE knocked out showed a 60% reduction of the plaque area. Another study (Pasini et al., 2007) found that MCP-1 and other inflammatory cytokines that were excessively expressed in patients with unstable angina might be related to the oxLDL-induced up-regulation of TLR4.

RNA interference technology was used to block TLR4 to investigate the function and mechanism of TLR4 in the oxLDL-induced up-regulation of LOX-1, MCP-1, and VCAM-1 expressions in HUVECs. Results showed that protein expressions of LOX-1, MCP-1, and VCAM-1 were significantly reduced. Thus, TLR4 had a key function in the oxLDL-induced up-regulation of protein expressions of LOX-1, MCP-1, and VCAM-1 in HUVECs.

NF- κ B, a type of multidirectional nuclear transcriptional regulatory factor, regulates the expression of proteins and genes related to inflammation, immunization, and growth regulation (Hayden and Ghosh, 2008). In its resting state, NF- κ B combines with its suppressor protein, I κ B, whereas it stays in the cytoplasm in its inactive state. After the TLR4 ligand combines with the TLR4 extracellular region, the signal is transferred to its adapter protein, MyD88, through the intracellular zone. MyD88 then interacts with interleukin-1 receptor-associated kinase (IRAK), which results in the auto-phosphorylation of IRAK (Li et al., 2005). Phosphorylated IRAK combines with and activates the tumor necrosis factor receptor-associated factor-6 (TRAF6) (Hacker et al., 2006), followed by the oligomerization of TRAF6. The signal is subsequently transferred to the NF- κ B-inducing kinase, which phosphorylates and degrades the I κ B kinase. This step is followed by the dissociation of NF- κ B from I κ B, the activation of NF- κ B, and quick nuclear translocation. The dissociated NF- κ B combines with a specific site in the gene operon to trigger gene transcription (An et al., 2010).

PDTC is a type of antioxidant that increases the permeability of cell membranes (Zhang et al., 2008). PDTC restrains I κ B degradation, and thus specifically inhibits NF- κ B activation. In this study, HUVECs were pretreated with PDTC and incubated with additional oxLDL for 48 h. NF- κ B expression was significantly reduced in the cell nucleus, which indicated that PDTC effectively restrained the nuclear translocation of NF- κ B. Simultaneously, protein expressions of LOX-1, MCP-1, VCAM-1, and TLR4 significantly decreased. This suggests that gene transcriptions of LOX-1, MCP-1, VCAM-1, and TLR4 are closely related to the nuclear translocation of NF- κ B. Bioinformatic studies (Baeuerle, 1991; Nagase et al., 1998) have shown that the gene promoter region of LOX-1, MCP-1, and VCAM-1 exists within the binding sites of NF- κ B, which indicates that gene transcription was regulated by NF- κ B. Based on results of the present study, the TLR4/NF- κ B signaling pathway can be concluded to be involved in the regulation of LOX-1, MCP-1, and VCAM-1 expressions in HUVECs. In addition, siTLR4 could inhibit the nuclear translocation of NF- κ B, which could also reduce the protein expression of TLR4. Thus, a positive feedback regulatory process of TLR4/NF- κ B/TLR4 in HUVECs exists under the influence of oxLDL.

After HUVECs were processed with siTLR4, gene silencing of TLR4 occurred after transcription, which resulted in a reduction in the protein expression of TLR4 at the cell surface. When TLR4 expression was reduced, the activation of downstream molecular NF- κ B was also relatively reduced, which affected LOX-1, MCP-1, and VCAM-1 expressions. After HUVECs were processed with PDTC, the activation of NF- κ B was restrained and the gene

transcriptions of LOX-1, MCP-1, VCAM-1, and TLR4 were reduced, which resulted in a reduction in protein expression. Therefore, the TLR4/NF- κ B signaling pathway is believed to have an important function in the oxLDL-induced up-regulation of LOX-1, MCP-1, and VCAM-1 expressions in HUVECs. The TLR4/NF- κ B signaling pathway could increase expressions of adhesion molecules, chemokines, and other inflammatory molecules to promote the adhesion and migration of white blood cells to endothelial cells. Therefore, the TLR4/NF- κ B signaling pathway is a very important target for the treatment of atherosclerosis. Although the knockout and silencing of TLR4 could effectively prevent the occurrence of atherosclerosis, the function of TLR4 must be examined closely. Because of its significance as a receptor in the innate immune system, blocking of the TLR4/NF- κ B signaling pathway might have a negative effect. More in-depth research and discussions are needed to study its specific mechanism and its potential applications for treating atherosclerosis.

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