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Original Article

Poly(ADP-ribose) polymerase 1 inhibition protects human aortic endothelial cells against LPS-induced inflammation response

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Atherosclerosis is a chronic inflammatory disease. Toll-like receptor 4 (TLR4) is an important signaling receptor and plays a critical role in the inflammatory response. Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme that can regulate the expression of various inflammatory genes. In this study, we investigated the role and the underlying mechanisms of PARP1 on lipopolysaccharide (LPS)-induced inflammation in human aortic endothelial cells. Compared with the control, LPS stimulation increased the protein expression of TLR4 and PARP1. TLR4 inhibition reduced LPS-induced upregulation of inducible nitric oxide synthase (iNOS) and ICAM-1 as well as PARP1. Nuclear factor κB (NF-κB) inhibition decreased ICAM-1 and iNOS expression. Inhibition of PARP1 decreased protein expression of inflammatory cytokines induced by LPS stimulation, probably through preventing NF-κB nuclear translocation. Our study demonstrated that LPS increased ICAM-1 and iNOS expression via TLR4/PARP1/NF-κB pathway. PARP1 might be an indispensable factor in TLR4-mediated inflammation after LPS stimulation. PARP1 inhibition might shed light on the treatment of LPS-induced inflammatory cytokines expression during atherosclerosis.

Keywords human aortic endothelial cell; atherosclerosis; inflammatory cytokine; toll-like receptor 4; poly(ADP-ribose) polymerase 1; nuclear factor κB

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Introduction

Atherosclerosis is a multifactorial progressive artery disease that can lead to cardiovascular disease, myocardial infarction, and stroke [1]. It is regarded as a chronic inflammatory process in which inflammation plays an important role from initiation through the phase of progression to ultimate complications [2].

Toll-like receptors (TLRs) are a family of type I transmembrane receptors that are expressed in a cell-type specific manner and can be activated by distinct pathogen-associated molecular patterns (PAMPs) [3]. TLRs contain a cytoplasmic domain that is homologous to the signaling domain of the interleukin 1 (IL-1) receptor [4]. Currently, more than 10 human TLRs have been discovered [5], but only TLR2 and TLR4 have been studied. TLR4, a key signaling receptor of innate immunity, can activate mitogen-activated protein kinase and nuclear factor κB (NF-κB) after lipopolysaccharide (LPS) stimulation, resulting in the activation of the adaptive immune response [5–7]. TLR4 also plays an important role in inflammation during atherosclerosis. Overexpression of TLR4 in human cell lines stimulates the expression of a variety of proinflammatory cytokines such as IL-1, IL-6 and IL-8 [8]. Faure *et al*. [9,10] have demonstrated that cultured human endothelial cells can express TLR4 under baseline conditions, and after stimulation with proinflammatory cytokines the levels of TLR2 and TLR4 are significantly increased. Edfeldt *et al*. [11] also found that the expression of TLR4 is markedly elevated in human atherosclerotic lesions and this kind of increase occurred preferentially in endothelial cells and macrophages.

Poly(ADP-ribose) polymerase 1 (PARP1) is a highly conserved nuclear enzyme that binds to DNA breaks as DNA damage sensor and signaling molecule. After binding to damaged DNA, PARP1 accomplishes its activation and auto-poly(ADP-ribosylation) that is pivotal for DNA repair [12]. However, excessive activation of PARP1 leads to intracellular depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP), driving cells into energy depletion, mitochondrial dysfunction and cell death [13]. On the other hand, PARP1 acts as a co-activator of the NF-κB to regulate the expression of a number of key inflammatory cytokines including inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1 (MCP-1), and adhesion molecules [14–16].

Considering the critical role of TLR4 and PARP1 in inflammation during atherosclerosis, we assumed that aortic

endothelial cells might express inflammatory cytokines via TLR4/PARP1/NF-κB pathway after LPS stimulation. Inhibition of PARP1 might protect against TLR4-mediated inflammation.

Materials and Methods

Cell culture and treatment

Human aortic endothelial cells (HAECs) were obtained from American Type Culture Collection (ATCC, Manassas, USA) and cultured in endothelial cell medium (ECM, ScienCell, San Diego, USA) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C up to passage 4. Before treatment or stimulation with reagents, the cells were serum starved for 24 h. HAECs were stimulated with LPS (100 ng/ml; Sigma-Aldrich, St Louis, USA) for 24 h. NF-κB inhibitor (BAY 11-7082, 1 μM; Sigma-Aldrich) was added into the medium 2 h before LPS stimulation [17].

siRNA transfection

The TLR4, PARP1 siRNA or negative control (NC) siRNA (GenePharma, Shanghai, China) was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) in 3 ml Opti-MEM (Invitrogen), and transfected into cells as described [18]. The Opti-MEM was replaced with ECM at 8 h after siRNA transfection. Then HAECs were stimulated by LPS.

Real-time polymerase chain reaction

Total RNA was isolated using Trizol (Invitrogen). RNA (1 μg) was reverse-transcribed for 1 h at 42°C using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada), in a volume of 20 μl. The reaction was terminated at 70°C for 5 min. After cDNA was generated, quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA). Each sample was analyzed in triplicate, and the expression was normalized to *GAPDH*. The primers for *TLR4* were as follows: forward, 5′-TGGATACGTTTCCTTATAAG-3′; reverse, 5′-GAAATGGAGGCACCCCTTC-3′; for *PARP-1*: forward, 5′-TTGAAAAAGCCCTAAAGGCTCA-3′; reverse, 5′-CTACTCGGTCCAAGATCGCC-3′; and for *GAPDH*: forward, 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse, 5′-TGTAGACCATGTAGTTGAGGTCA-3′. Amplification, detection, and data analysis were performed with the iCycler qRT-PCR system (Bio-Rad Laboratories).

Western blot analysis

The proteins were extracted from cells with the RIPA lysis buffer (Beyotime, Nantong, China) and assayed by the BCA protein assay kit (Beyotime). Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore, Billerica, USA). Then the membranes were blocked with 5% non-fat milk for 2 h at room temperature. The nitrocellulose membranes were probed with goat anti-TLR4 antibody (1 : 250; R&D Systems, Minneapolis, USA), rabbit anti-PARP1 antibody (1 : 100; Sigma-Aldrich), mouse anti-ICAM-1 antibody (1 : 500; Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-iNOS antibody (1 : 1000; Cell Signaling Technology, Beverly, USA), rabbit anti-p65 NF-κB antibody (1 : 1000; Cell Signaling Technology) and rabbit monoclonal anti-phospho-NF-κB p65 (p-p65, 1 : 1000; Cell Signaling Technology) at 4°C overnight. Rabbit anti-β-actin antibody (1 : 1000; Cell Signaling Technology) was used as the loading control. After being washed in TBST (50 mM Tris; 150 mM Nacl; 0.05% Tween 20; pH 7.6) 10 min for three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 5000; ZSGB-BIO, Beijing, China) for 2 h. The proteins were visualized with an enhanced chemiluminescence detection kit (Millipore) and analyzed by Image-Pro Plus 6.0.

Immunofluorescence

The protein expression of p-p65 NF-κB was determined by immunofluorescence. Briefly, cells were fixed in 4% paraformaldehyde. After being blocked with BSA for 30 min, samples were incubated with mouse monoclonal anti-p-p65 NF-κB (1 : 100; Cell Signaling Technology) overnight at 4°C. After samples were washed in TBS 10 min for three times, Alexa 488-conjugated goat anti-mouse IgG (1 : 500; Jackson immunoresearch, West Grove, USA) was used as secondary antibodies. A drop of Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, USA) was used to seal the coverslip. Images were assessed by laser scanning confocal microscopy (LSM710, Carl Zeiss, Ostalbkreis, Germany). Data were analyzed by using Image-Pro Plus 6.0.

Statistical analysis

All data are presented as mean ± standard deviation (SD). SPSS v16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Intergroup comparisons involved the two-tailed Student *t* test or one-way analysis of variance followed by Tukey test (with equal variances assumed) or Dunnett T3 test (with equal variances not assumed). $P < 0.05$ was considered to be statistically significant.

Results

LPS increased the expression of TLR4 and PARP1

After HAECs were stimulated with LPS (100 ng/ml) for 24 h, the protein expression of TLR4 and PARP1 was

determined by western blot analysis. Compared with control, TLR4 expression increased 2.54 ± 0.25 fold and PARP1 expression increased 2.33 ± 0.19 fold (**Fig. 1**), which indicated that TLR4 and PARP1 might take part in LPS-induced inflammation.

TLR4 inhibition decreased LPS induced PARP1 up-regulation

Then we investigated the role of TLR4 in LPS-induced inflammation by using TLR4 siRNA. The results of qRT-PCR and western blot showed that TLR4 siRNA significantly decreased the expression of TLR4 at the mRNA and protein level to 0.29 ± 0.08 and 0.20 ± 0.08 compared with the control group, respectively [**Fig. 2(A–C)**], which meant that the TLR4 siRNA was effective, while the NC of TLR4 siRNA (si-NC) had no effect on it.

We then investigated the effect of TLR4 on PARP1 expression. As we expected, compared with the LPS group, the protein expression of PARP1 was significantly decreased to 0.48 ± 0.06 [**Fig. 2(D,E)**] when TLR4 was inhibited by siRNA. These results indicated that TLR4 played a critical role in LPS-induced PARP1 expression.

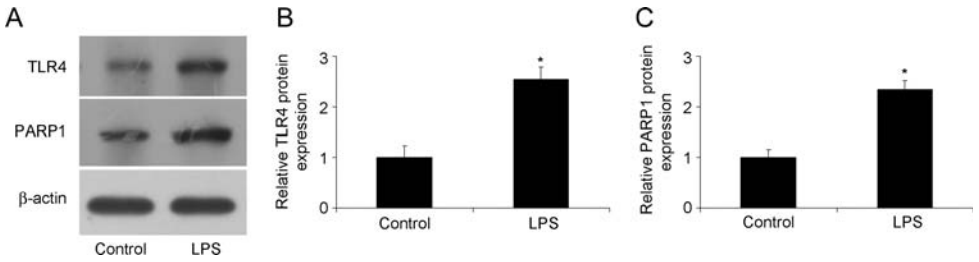

Figure 1 LPS stimulation increased protein expression of TLR4 and PARP1 After HAECs were stimulated by 100 ng/ml LPS for 24 h, the expression of TLR4 and PARP1 was assessed by western blot analysis. (A–C) Western blot analysis of TLR4 and PARP1 expression. Control: cells without stimulation. Values are expressed as mean ± SD from four separate experiments. $^{*}P < 0.05$ vs. control.

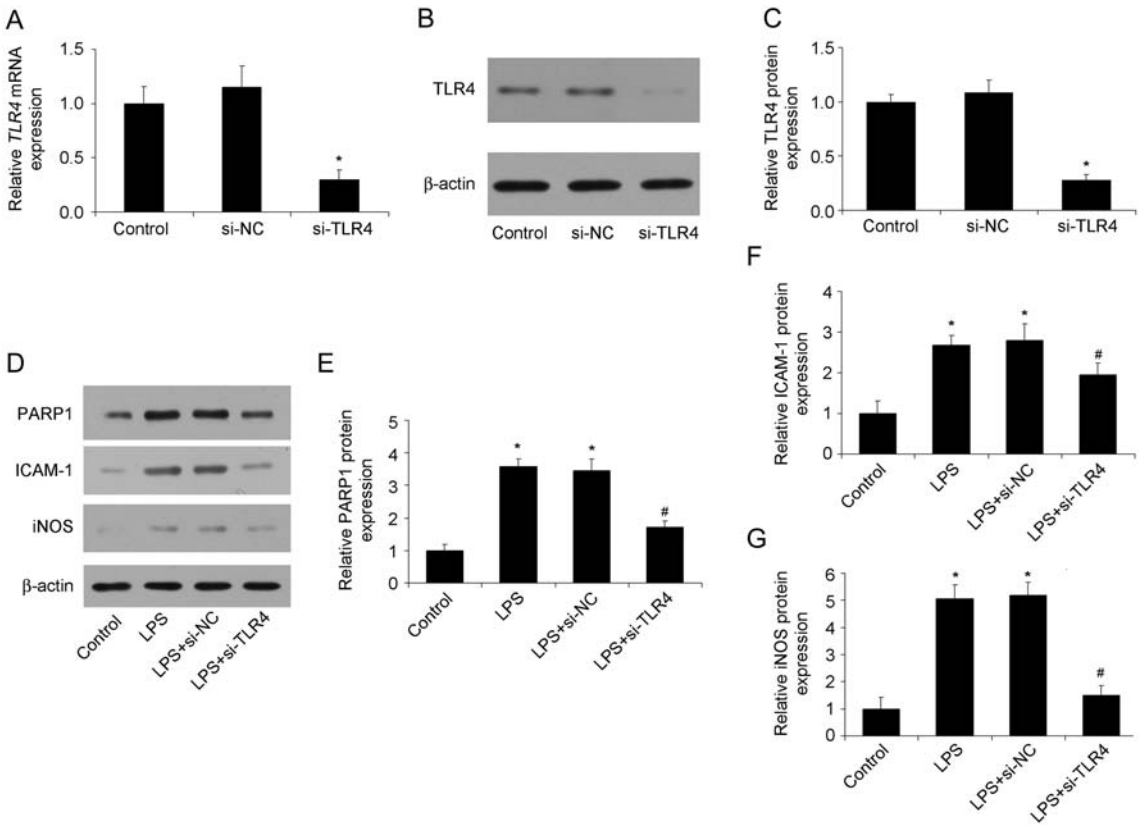

Figure 2 TLR4 inhibition reduced LPS-induced protein expression of PARP1 and inflammatory cytokines The mRNA and protein expression of TLR4 was determined by RT-PCR and western blot analysis after TLR4 siRNA transfection. After TLR4 was inhibited by siRNA, the protein expression of PARP1, ICAM-1, and iNOS was determined after cells were stimulated by LPS for 24 h. (A) RT-PCR analysis of *TRL4* mRNA expression. (B, C) Western blot analysis of protein expression of TLR4. (D–G) Western blot analysis of protein expression of PARP1, ICAM-1 and iNOS. si-NC, negative control of siRNA of TLR4; si-TLR4, siRNA of TLR4. Control: cells without stimulation. Values are expressed as mean ± SD from three separate experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

TLR4 inhibition reduced LPS-induced ICAM-1 and iNOS expression

When TLR4 was inhibited, the protein expression of ICAM-1 and iNOS was detected. Western blot analysis showed that LPS stimulation could upregulate protein expression of ICAM-1 and iNOS [2.68 ± 0.24 and 5.05 ± 0.50, **Fig. 2(D,F,G)**]. However, the protein expression of ICAM-1 and iNOS was decreased to 0.73 ± 0.11 and 0.29 ± 0.07 by TLR4 siRNA [**Fig. 2(D,F,G)**], while the NC had no effect on it. These results suggested that elevated expression of inflammatory cytokines induced by LPS was dependent on TLR4.

PARP1 inhibition reduced ICAM-1 and iNOS expression induced by LPS

To ensure that effective PARP1 inhibition was achieved in target cells, we analyzed mRNA and protein expression of PARP1 after PARP1 inhibition. The results of qRT-PCR and western blot showed that TLR4 siRNA significantly decreased the expression of TLR4 at the mRNA and protein level to 0.34 ± 0.05 and 0.26 ± 0.04 compared with the control group, respectively [**Fig. 3(A–C)**].

When PARP1 was inhibited by PARP1 siRNA, cells were stimulated with LPS for 24 h, and the expression of inflammatory cytokines was analyzed by western blot analysis. The results demonstrated that protein expression of ICAM-1 and iNOS was decreased to 0.33 ± 0.09 and 0.35 ± 0.08 by PARP1 inhibition [**Fig. 3(D–F)**], while the NC of PARP1 siRNA had no effect, which indicated PARP1 was indispensable for ICAM-1 and iNOS expression induced by LPS.

NF-κB inhibition decreased LPS-induced ICAM-1 and iNOS expression

As reported previously, BAY 11-7082, an inhibitor of NF-κB, strongly could suppress the translocation and activation of NF-κB. To explore the role of NF-κB in ICAM-1 and iNOS expression, we inhibited NF-κB activity by using BAY 11-7082. After NF-κB activity was inhibited by BAY 11-7082, cells were stimulated by LPS. Compared with the LPS group, the protein expression of ICAM-1 and iNOS was significantly decreased after NF-κB inhibition (**Fig. 4**), which indicated that NF-κB played a critical role in ICMA-1 and iNOS expression.

PARP1 down-regulation reduced inflammatory cytokines expression by preventing NF-κB nuclear translocation

Considering the critical role of NF-κB on iNOS and ICAM-1 expression [15], we speculated that the negative effect of PARP1 inhibition was associated with defective NF-κB activation. **Figure 5(A)** showed that p-p65 NF-κB was mostly cytoplasmic in the control group, but its localization quickly changed to the nucleus after LPS stimulation. However, p-p65 NF-κB remained primarily cytoplasmic with LPS treatment when PARP1 was inhibited. Then

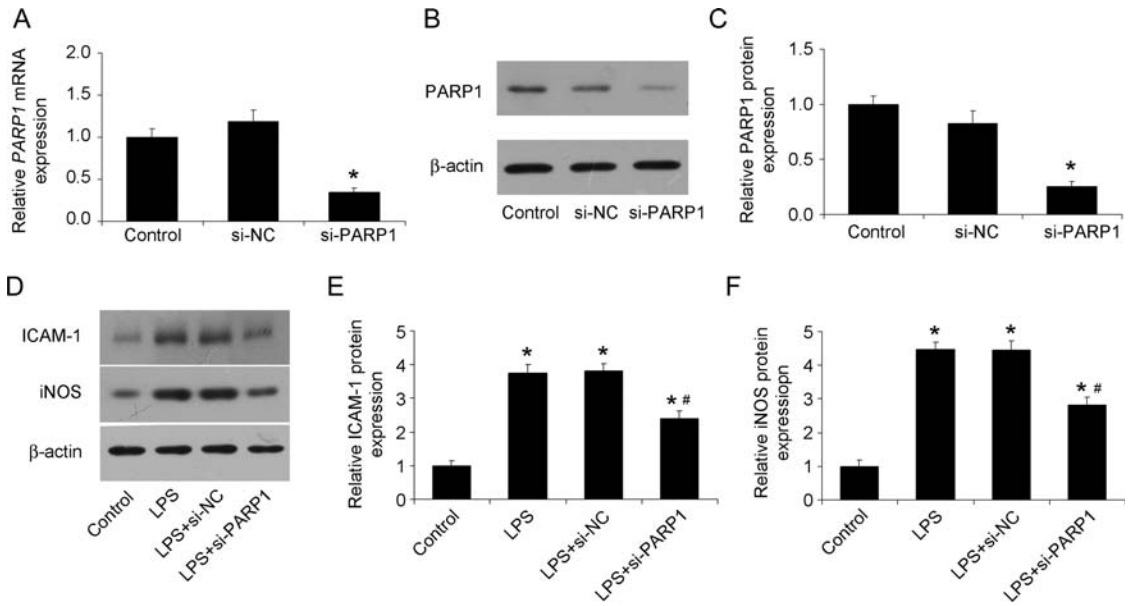

Figure 3 PARP1 inhibition reduced LPS-induced protein expression of ICAM-1 and iNOS The mRNA and protein expression of PARP1 was determined by RT-PCR and western blot analysis after PARP1 siRNA transfection. After PARP1 was inhibited by siRNA, the protein expression of ICAM-1 and iNOS was determined after cells were stimulated by LPS for 24 h. (A) RT-PCR analysis of *PARP1* mRNA expression. (B, C) Western blot analysis of protein expression of PARP1. (D–F) Western blot analysis of protein expression of ICAM-1 and iNOS. si-NC, negative control of siRNA of PARP1; si-PARP1, siRNA of PARP1. Control: cells without stimulation. Values are expressed as mean ± SD from three separate experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

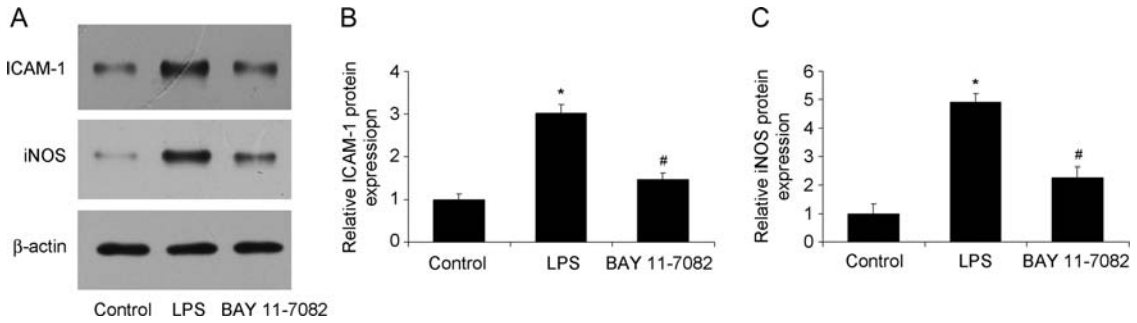

Figure 4 NF-κB inhibition reduced the protein expression of ICAM-1 and iNOS After NF-κB inhibition by BAY 11-7082 (1 μM), cells were stimulated by LPS, then the protein expression of ICAM-1 and iNOS was determined by western blot analysis. (A–C) Western blot analysis of protein expression of ICAM-1 and iNOS. BAY 11-7082, NF-κB inhibitor; control: cells without stimulation. Values are mean ± SD from two separate experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

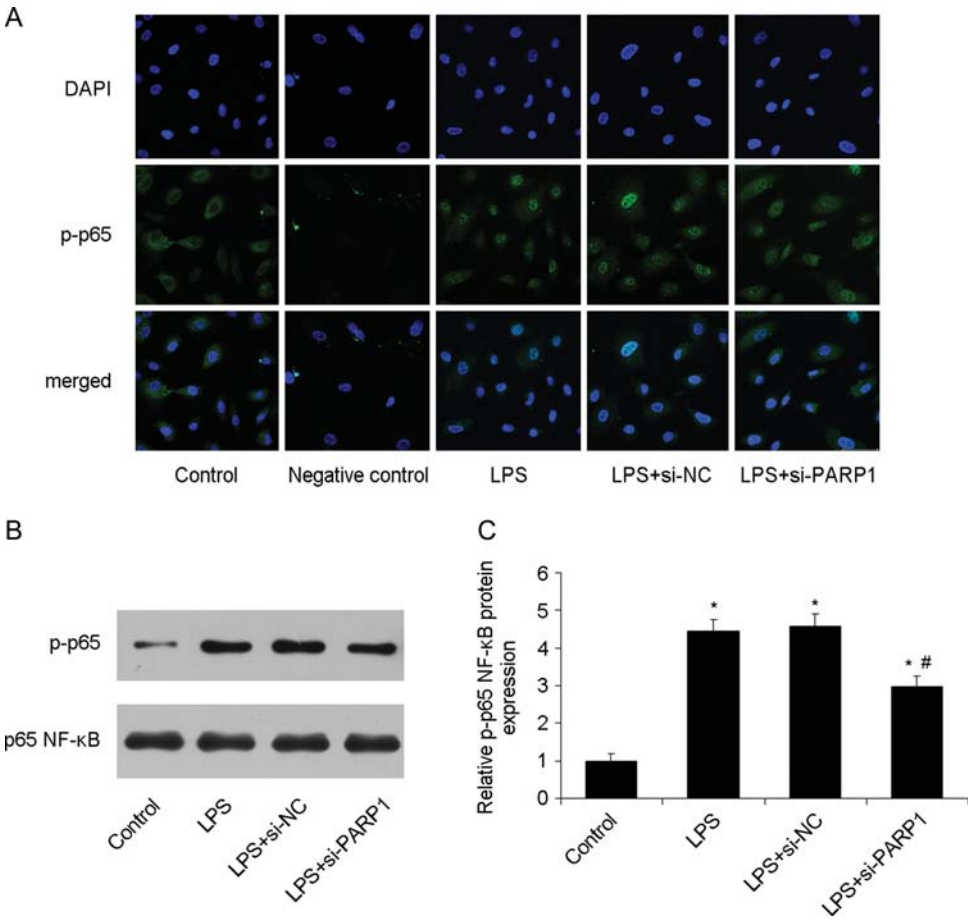

Figure 5 PARP1 inhibition suppressed NF-κB nuclear translocation in HAECs After PARP1 was inhibited by siRNA, p-p65 expression was determined by immunofluorescence and western blot analysis. (A) Immunofluorescence analysis of p-p65 NF-κB (green) and DAPI (blue). Nuclei were labeled with DAPI (blue); p-p65 NF-κB was stained with mouse anti-p-p65 NF-κB primary antibody and Alexa 488-conjugated goat anti-mouse second antibody (green). (B, C) Western blot analysis of protein expression of p-p65 NF-κB. Control, cells without stimulation. Values are mean ± SD from three separate experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

we measured protein expression of p-p65 NF-κB. Compared with the control, LPS could significantly increase p-p65 NF-κB expression to 4.45 ± 0.29 [**Fig. 5(B,C)**], while PARP1 siRNA reduced it to 2.98 ± 0.27 [**Fig. 5(B,C)**]. These results indicated that PARP1 inhibition could suppress the nuclear translocation and activation of NF-κB.

Discussion

In this study, we examined the role and the underlying mechanism of PARP1 in TLR4-mediated inflammation after LPS stimulation in HAECs. We found that LPS stimulation increased the protein expression of TLR4 and

PARP-1. TLR4 inhibition reduced LPS-induced upregulation of iNOS and ICAM-1. PARP1 inhibition could decrease TLR4-mediated inflammation after LPS stimulation via inhibition of NF-κB nuclear translocation. It might suggest that PARP1 inhibition was an effective method to alleviate the TLR4-mediated inflammation after LPS stimulation.

TLR4, a member of TLR family, is a type I transmembrane protein that has recently been shown to be associated with the innate immune response and cellular activation in response to microbial antigens [19–21]. TLR4 also is proved to be the primary signaling receptor for LPS [22,23]. Hoshino *et al*. [24] showed that the TLR4 knockout mice failed to respond to LPS. It is now becoming clear that TLR4 not only serves as a receptor for PAMPs but also takes part in the initiation and progression of cardiovascular disease. Considering the important role of TLR4 in atherosclerosis, we investigated the expression of TLR4 after LPS stimulation in HAECs. We found that LPS stimulation increased TLR4 protein expression, which suggested that TLR4 was a receptor for LPS and played an important role in LPS stimulation.

Inflammation and inflammatory cytokines play important roles in the development and progression of atherosclerosis [25]. Intercellular adhesion molecule 1 (ICAM-1) can mediate the margination, adhesion and transendothelial migration of circulating mononuclear cells. In addition, ICAM-1 can activate mononuclear cells to release matrix metalloproteinases, decrease the plaque stability and promote plaque rupture [26]. iNOS can lead to excessive production of nitric oxide and oxidative damage to vascular tissue. In our experiment, LPS stimulation significantly increased the protein expression of ICAM-1 and iNOS as well as TLR4. However, the expression of these inflammatory cytokines was significantly decreased after TLR4 downregulation, which suggested that LPS stimulation increased ICAM-1 and iNOS expression via TLR4. TLR4 played a pivotal role in LPS-induced inflammation. We also found that LPS could increase protein expression of PARP1 and TLR4 inhibition could reduce the upregulation of PARP1.

PARP1 is an abundant nuclear enzyme that accomplishes the transfer of ADP-ribose from their substrate β-NAD^+ to itself and other nuclear chromatin-associated proteins [27,28]. Inhibition of PARP1 by gene knockout or inhibitors demonstrates a critical role of PARP1 in inflammatory processes such as myocardial infarction, diabetes-induced endothelial dysfunction, and LPS-induced septic shock [29–31]. In addition, PARP1 is also a co-activator of NF-κB in NF-κB-mediated transcription of various key inflammatory cytokines [32,33], such as iNOS and ICAM-1. Considering the critical functions of PARP1, we then investigated the underlying role of PARP1 in TLR4-mediated inflammation. PARP1 inhibition by siRNA decreased the expression of iNOS and ICAM-1 after LPS stimulation, which suggested that PARP1 was indispensable in LPS-induced inflammation. We further explored the possible mechanisms. As there were close associations among PARP1, NF-κB, and inflammatory cytokines, we detected NF-κB expression and nuclear translocation. As we expected, p-p65 NF-κB was strictly cytoplasmic before LPS stimulation, but it quickly shifted from cytoplasm to nuclear after cells were stimulated by LPS. However, it remained primarily cytoplasmic during the course of treatment with LPS after PARP1 inhibition. We could demonstrate that PARP1 exerted a critical role by promoting p-p65 NF-κB nuclear translocation. PARP1 was a determining factor in TLR4-mediated inflammation in endothelial cells after LPS stimulation.

For the potential mechanism by which PARP1 regulates NF-κB-mediated gene expression, several studies have focused on factors that are related directly to NF-κB DNA binding to the promoter regions of target genes. Our results also showed that PARP1 was critical for efficient nuclear translocation of the transcription factor. However, whether PARP1 is the only defining factor in NF-κB nuclear translocation and expression of target genes needs further investigation. In addition, although the mechanism by which PARP1 affects the activation and nuclear translocation of NF-κB is not understood, PARP1 has been reported to interact with several kinases such as DNA-dependent protein kinase and ERK [34], which may shed light on a new direction of research.

Funding

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