GEF-H1-RhoA signalling pathway mediates LPS-induced NF-κB transactivation and IL-8 synthesis in endothelial cells

Feng Guo[1], Jiajun Tang[1], Zengding Zhou[1], Yi Dou[1], Derek Van Lonkhuyzen[2], Chengjin Gao[3], Jingning Huan[1].*

1 Department of Burn and Plastic Surgery, Shanghai Jiao Tong University, School of Medicine, Rui Jin Hospital, Shanghai, China.

2 Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Brisbane, Australia.

3 Department of Intensive Care Unit, Shanghai Tenth People's Hospital, Shanghai, China.

***Corresponding author:** Jingning Huan.

Address: Department of Burn and Plastic Surgery, Shanghai Jiao Tong University, School of Medicine, Rui Jin Hospital, Shanghai, 230022, China.

Telephone: +86-21-3413638

E-mail address: friendshiper@live.cn

Abstract

Secretion of proinflammatory cytokines by LPS activated endothelial cells contributes substantially to the pathogenesis of sepsis. However, the mechanism involved in this process is not well understood. In the present study, we determined the roles of GEF-H1 (Guanine-nucleotide exchange factor-H1)-RhoA signalling in LPS-induced interleukin-8 (IL-8, CXCL8) production in endothelial cells. First, we observed that GEF-H1 expression was upregulated in a dose- and time-dependent manner as consistent with TLR4 (Toll-like receptor 4) expression after LPS stimulation. Afterwards, Clostridium difficile toxin B-10463 (TcdB-10463), an inhibitor of Rho activities, reduced LPS-induced NF-κB phosphorylation. Inhibition of GEF-H1 and RhoA expression reduced LPS-induced NF-κB and p38 phosphorylation. TLR4 knockout blocked LPS-induced activity of RhoA, however, MyD88 knockout did not impair the LPS-induced activity of RhoA. Nevertheless, TLR4 and MyD88 knockout both significantly inhibited transactivation of NF-κB. GEF-H1-RhoA and MyD88 both induced significant changes in NF-κB transactivation and IL-8 synthesis. Co-inhibition of GEF-H1-RhoA and p38 expression produced similar inhibitory effects on LPS-induced NF-κB transactivation and IL-8 synthesis as inhibition of p38 expression alone, thus confirming that activation of p38 was essential for the GEF-H1-RhoA signalling pathway to induce NF-κB transactivation and IL-8 synthesis. Taken together, these results demonstrate that LPS-induced NF-κB activation and IL-8 synthesis in endothelial cells are regulated by the MyD88 pathway and GEF-H1-RhoA pathway.

Keywords: Lipopolysaccharide; Guanine-nucleotide exchange factor; RhoA; p38; nuclear factor-κB; Interleukin-8.

1. Introduction

Severe sepsis or septic shock is a common and serious consequence of gram-negative bacterial infection, which accounts for high mortality in critically ill patients (Wendel et al., 2007). Endothelial cells line the inner wall of blood vessels, helping maintain organ homeostasis. Exposed to blood flow, endothelial cells are the primary targets for inflammatory agents during local and systemic inflammation (Cook-Mills and Deem, 2005). The bacterial endotoxin lipopolysaccharide (LPS), the major component of the outer surface of Gram-negative bacteria, induces a multitude of endothelium dysfunctions, including up-regulation of adhesion molecules, disorder of procoagulant activity, enhanced endothelial permeability, and over-expression of proinflammatory mediators (Aird, 2003). Endothelial cells express chemokines that initiate the activation and recruitment of circulating leukocytes at sites of tissue inflammation (Ait-Oufella et al., 2010). Among these activities, secretion of interleukin-8 (IL-8, CXCL8) by LPS-activated endothelial cells contributes substantially to the development of inflammation (Baggiolini et al., 1997). IL-8 displays a chemotactic activity for neutrophils, which are the first line of immune cells to be recruited to inflamed areas (Smart and Casale 1993). IL-8 also induces neutrophils to synthesize several toxic products, such as arachidonic acid metabolites (Wertheim et al., 1993). Thus, during septicemia, IL-8 participates in a series of cellular pathological events that severely injure endothelium and surrounding tissues. However, the precise mechanism of LPS-induced signaling that leads to IL-8 secretion in endothelial cells is poorly understood. Clarification of this pathway may be of immense significance in the design of anti-inflammatory therapies that act by regulating chemokine responses leading to septic shock-related events.

The Rho family of small GTPases (Rho-GTPases) act as molecular switches to control many basic cellular activities that are also critical to the specialized cellular functions, such as actin cytoskeleton organization, cell migration and adhesion, reactive oxygen species formation, and apoptosis (Hall, 1994; Fiorentini et al., 2003; Riento and Ridley, 2003; Doe et al., 2007). Moreover, some studies have implicated RhoA, Rac1 and Cdc42 in signalling pathways emanating from TLR2, TLR4, TLR3, and TLR9 (Kim and Woo, 2002; Liu-Bryan et al., 2005). The Rho-GTPases might participate in another potential mechanism for regulating TLR signal induction (Ruse and Knaus, 2006). NF-κB is involved in the control of a variety of genes activated upon inflammation including the promoter region of the IL-8 gene, NF-κB plays a central role in the inflammatory response to infection and tissue injury (Yasumoto et al., 1992). However, studies with TcdB-10463, an inhibitor of Rho proteins activities, demonstrated strong anti-inflammatory effects by inhibiting NF-κB-translocation and activation in human endothelial cells (Hippenstiel et al., 2000), suggesting a potential role of Rho proteins in promoting LPS-induced inflammation (Perona et al., 1997).

However, the activation of Rho-GTPases is specifically regulated by several members of Guanine-nucleotide exchange factors (GEFs) (Zheng, 2001). GEF-H1 has been characterized as a Rho-specific GEF that localizes on microtubules (MT) and exhibits Rho-specific GDP/GTP exchange activity (Ren et al., 1998). In its MT-bound state, the guanosine-exchange activity of GEF-H1 is suppressed, whereas GEF-H1 release from MT stimulates Rho-specific GEF activity (Bokoch et al., 2002). LPS-induced TLR-mediated NF-κB-translocation and activation are inhibited by GEF-deficientcy (Shibolet et al., 2007). Although the expression of GEF-H1 and RhoA have been identified in endothelial cells, very few reports have focused on their inflammatory roles

in these cells. In view of previous studies that link GEF-H1-RhoA signalling with inflammation, our present study evaluated their roles in mediating IL-8 secretion in LPS-stimulated endothelial cells.

In this study, we have shown that GEF-H1 and RhoA mediate LPS-induced NF-κB transactivation and IL-8 expression via interaction between GEF-H1 and TLR4.

2. Material and methods

2.1 Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 U/ml streptomycin in a humidified 37℃, 5% CO_2 incubator. DMEM and FBS were from GIBCO BRL (Mississauga, ON). Antibodies used in Western blotting for human GEF-H1, RhoA, p65, phospho-p65^{Ser536}, phosphor-p38, p38 and MyD88 were obtained from Cell Signalling Technology. Antibodies used in Western blotting for TLR4 were purchased from Santa Cruz Biotechnology.

2.2. Western blot assay

Cultured HUVECs (3-4 $\times 10^6$) were washed twice with PBS, lysed and scraped off the dishes, transferred to a 1.5-ml tube, and centrifuged. Nuclear proteins were extracted with NE-PER^{TM} nuclear and cytoplasmic extraction regents (Pierce Chemical Co., NY, USA) according to manufacturer's instructions. The pellet was resuspended in SDS sample buffer and heated at 99 °C for 5 min. The eluted proteins were applied to SDS-polyacrylamide gels and electrotransferred to nitrocellulose transfer membranes (BioScience). The membranes were blocked for 2 h in 2% bovine serum albumin in TBST (20 mM Tris-HCl [pH 7.6], 0.15 M sodium chloride, and 0.1% Tween 20), and then incubated with primary antibodies in TBST overnight. After washing three times with TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or -rabbit immunoglobulin (Amersham Pharmacia Biotech) diluted 1:2000 in TBST. After three washes in TBST, the blot was developed with the enhanced chemiluminescence system (Millipore), according to the manufacturer's instructions.

2.3. Small interfering RNA (siRNA) transfection

Stealth™ RNAi duplexes, against human GEF-H1, RhoA, MyD88, and p38, were obtained from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA). All sets of RNAi molecules were transfected individually into HUVECs at a 50 nM concentration using Lipofectamine 2000 (Carlsbad, CA) according to the manufacturer's instructions. Duplex siRNA were constructed against sequences coding for GEF-H1 positions 998-1022 (5'-AGA ACU GGC UGA UGA GCA GAU CAC C-3'), the target sequence of siRNA for RhoA corresponding to positions 319-343 (5'-UGA GCA AGC AUG UCU UUC CAC AGG C-3'), the target sequence of siRNA for MyD88 (5'-CCG GAU GGU GGU GGU UGU CUC UGA U-3'), the target sequence of siRNA for TLR4 (5'- GGG TAA GGA ATG AGC TAG TAA AGA A-3'), and for p38 (5'-GCU GAC AUA AUU CAC AGG GAC CUA A-3'). A scrambled negative control siRNA (5'-UUC UCC GAA CGU GUC ACG U-3') was also included. The abilities of the RNAi molecules to knockdown target proteins expression were analyzed at 48 h after transfection by western blot analysis.

2.4. Determination of RhoA activation by pull-down assay

RhoA-GTP was assayed by adapting published protocols (Ren and Schwartz, 2000). Briefly, proteins were extracted for 3 min at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.65 M NaCl, 10 mM MgCl2 , 10 µg/ml leupeptin, 10µg/ml aprotinin, 1 mM PMSF; 0.7 ml). Clarified extracts were incubated for 20 min at 4 °C with glutathione-Sepharose (Amersham Biosciences) coupled to bacterially expressed GST-Rho binding domain of rhotekin (RBD; 30 µg) and then washed five times with lysis buffer. Samples precipitated from 200 µg of total protein were resolved by 12.5% SDS-PAGE, and bound RhoA-GTP was visualized by Western blotting with anti-RhoA antibody (Cell Signaling).

2.5. Electrophoretic Mobility Shift Assay (EMSA)

EMSA for NF-κB was performed using the Lightshift Chemiluminescent EMSA kit (Pierce) following the manufacturer's protocol. Briefly, DNA was biotin labeled using the biotin 3'-end-labeling kit (Pierce) in 50 µl reaction buffer and 5 pmol of double-stranded NF-κB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5') incubated in a microfuge tube with 10µl of 5× terminal deoxynucleotidyltransferase buffer, 5µl of 5µM biotin-N4-CTP, 10 units of diluted terminal deoxynucleotidyltransferase, and 25µl of ultrapure water at 37°C for 30 min. The reaction was stopped with 2.5µl of 0.2 M EDTA. To extract labeled DNA, 50µl of chloroform:isoamyl alcohol (24:1) were added to each tube and centrifuged at 13,000 ×g. The top aqueous phase containing the labeled DNA was further used for binding reactions. Each binding reaction contained 1×binding buffer [100 mM Tris, 500 mM KCl, and 10 mM DTT (pH 7.5)], 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/µl poly (deoxyinosinic-deoxy-cytidylic acid), 0.05% NP40, 3 µg of nuclear extract, and 20 fmol of biotin-end-labeled target DNA. The contents were incubated at room temperature for 20 min. To this reaction mixture, 5µl of 5× loading buffer was added, subjected to gel electrophoresis on a native polyacrylamide gel, and transferred to a nylon membrane. After transfer was completed, DNA was cross-linked to the membrane using an UV cross-linker equipped with a 254 nm bulb. The biotin-end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate according to the manufacturer's instructions.

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR) assay

Total RNA was prepared from HUVEC cells with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. First-strand cDNA was synthesized using oligo (dT) 15 primer and M-MLV reverse transcription technique (Promega, Madison, WI) on 4 µg of total RNA in 40 µL reaction volume. Taq DNA Polymerase was used to amplify the resulting cDNA with a 25 µL reaction volume. The primer sequences were designed as follows: the 223-bp human GEF-H1 product was amplified using the following sequences: 5'-ACA CGC TTC CTC AGC CAG CTA TTA-3' and 5'-AAT TGC TGG AAG CGT TTG TCT CGG-3'; the 283-bp human IL-8 product using the following primers: 5'-ATG ACT TCC AAG CTG GCC GT-3'and 5'-CCT CTT CAA AAA CTT CTC CAC ACC-3'; and the 300-bp GAPDH product using the following primers: 5'-GGT CTA CAT GGC AAC TGT GA-3' and 5'-ACC AGG TGG TCT CCT CTG A-3'. PCR products were seen on 2% agarose gels, following electrophoresis by ethidium bromide staining, and photographed under ultraviolet (UV) light.

2.7. Quantitation of IL-8 by Enzyme linked immunosorbent assay (ELISA)

The supernatants previously prepared were assayed for IL-8 protein content using commercial enzyme linked immunosorbent assay (ELISA) kits for IL-8 (BioSciences, USA) according to the manufacturer's instructions. Briefly, each well of a 96-well plate was coated overnight with capture antibody before being washed with PBS containing 0.05% Tween; then test supernatant was added to the appropriate wells. After being incubated for 1 h at room temperature, the wells were washed, and a detection antibody was added and incubated for 1 further h at room temperature. The wells were then washed with PBS/Tween, and horseradish peroxidase-conjugated streptavidin was added before being incubated for 1 h at room temperature. Finally, the color was developed by adding peroxidase substrate to each well before reading the absorbance at 450 nm using a Dynatec plate reader.

2.8. Statistical analysis

All data are expressed as mean ± SEM. Student's t-test was used for all statistical analysis. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. LPS up-regulates expression of GEF-H1

As GEFs are regulators of multiple cell functions and may be involved in NF-κB activation, their roles originating from the activated LPS-TLR signalling pathway was investigated. HUVECs were treated with various concentrations of LPS (0.1ng/ml-10μg/ml) for 24 h. Dose-dependent up-regulation of protein and mRNA expression was detected by Western blot and RT-PCR (Fig. 1A and 1B). The expression of GEF-H1 protein and mRNA exhibited an increasing trend with alteration of the concentration of LPS. The curve peaked at a concentration of 100ng/ml compared with the control group ($p<0.01$). Subsequently, HUVECs were incubated with LPS (100ng/ml) at different indicated times. Immunoblot and RT-PCR analysis revealed that LPS up-regulated GEF-H1 expression in a time-dependent manner, peaking at 3 h after stimulation (Fig.1C and 1D). Interestingly, TLR4 expression was also up-regulated in a dose- and time-dependent manner as consistent with GEF-H1 expression after LPS stimulation.

3.2. GEF-H1-RhoA signalling is required for LPS-induced NF-κB transactivation

Exposure of endothelial cell monolayers to LPS (100 ng/ml) resulted in a time-dependent increase of cytosolic NF-κB phosphorylation, and the maximum level of phosphorylation of cytosolic NF-κB appeared at 40 min after LPS stimulation (Fig 2A). Endothelial cells were preincubated with 0.01 to 100 ng/ml of TcdB-10463 (inhibitor of RhoA, Cdc42, and Rac, but not other members of the Ras surperfamily of small GTP-binding proteins) for 60 minutes prior to stimulation with 100 ng/ml LPS for 40 minutes. The TcdB-10463 reduced LPS-related phosphorylation of NF-κB at Ser 536 in a dose-dependent manner. Although TcdB-10463 could not completely block phosphorylation of NF-κB at Ser 536, these data indicated that Rho proteins were required for LPS-related phosphorylation of NF-κB in endothelial cells. (Fig 2B)

To examine the effects of GEF-H1 and RhoA on LPS-induced NF-κB activation in more detail, we used GEF-H1 and RhoA specific siRNAs to observe changes in LPS-induced NF-κB activation. HUVEC cells

were transfected with GEF-H1 siRNA or RhoA siRNA or control siRNA for 48 hours, then treated with TcdB-10463 for 1 hour prior to LPS stimulation (100 ng/ml for 40 minutes). The inhibitory effects of specific siRNAs on target proteins were evaluated by Western blot (Fig 3A). The results presented in Fig 3B and 3C demonstrate that LPS treatment triggered more apparent augment of NF-κB phosphorylation at Ser 536 in the nucleus than LPS-untreated ECs. However, transfection alone with either GEF-H1 or RhoA siRNA both substantially impeded LPS-induced phosphorylation of NF-κB at Ser 536. In addition, co-transfection of GEF-H1 and RhoA siRNAs blocked LPS-induced phosphorylation of NF-κB to a similar extent as their transfection alone. Remarkably, these data indicate essential roles of GEF-H1 and RhoA in mediating LPS-induced phosphorylation of NF-κB.

3.3. GEF-H1-RhoA signalling regulates LPS-induced NF-κB transactivation and IL-8 expression through TLR4

To determine whether TLR4 mediates LPS-induced RhoA activity, HUVECs were transiently transfected with TLR4 siRNA or unspecific siRNA (control siRNA). After a 48-h incubation, cells were stimulated with medium alone or LPS (100 ng/ml) and then harvested. The ability of TLR4 siRNA to inhibit protein expression was analysed by Western blot (Fig. 4A). RhoA activity was detected by a cellular GTP-Rho assay, NF-κB DNA binding activity was monitored by EMSA (Fig. 4B). In endothelial cells overexpressing TLR4 (either treated with or without LPS), was effectively inhibited by TLR4-specific siRNA. LPS significantly enhanced activation of RhoA into GTP-RhoA and NF-κB transactivation by a time-dependent manner, however, LPS-induced RhoA activation and NF-κB transactivation were significantly inhibited by transfection of TLR4-specific siRNA at different indicated times. These results suggest that LPS, acting through TLR4, subsequently stimulates RhoA activation and induces NF-κB transactivation.

Indeed, previous studies have demonstrated that MyD88 is a key adaptor molecule and plays a crucial role in TLR-mediated signalling pathways (Kawai et al., 1999). To investigate whether MyD88 is an upstream regulator of RhoA activation, we used endothelial cells transfected with MyD88 siRNA to analyse LPS-stimulated activation of RhoA. Interestingly, stimulation by LPS caused no detectable difference in the activation of RhoA amongst the indicated times, demonstrating that MyD88 is not required for LPS-induced activation of RhoA (Fig. 4C). However, inhibition of MyD88 effectively blocked NF-κB DNA binding activity (Fig. 4D). These results suggest that MyD88 plays a role in the regulation of LPS-induced NF-κB transactivation, but does not effect RhoA activation.

To further confirm the above results, HUVECs were transfected with GEF-H1, RhoA, MyD88 or control siRNA for 48 hours, then exposed to medium or LPS (100 ng/ml) for 40 minutes. After control siRNA transfection, the cells treated with medium or LPS were used as negative control and positive control respectively. Therefore, compared with the positive control, LPS-induced phosphorylation and DNA binding activity of NF-κB in nucleus was significantly inhibited by individual transfection of GEF-H1, RhoA or MyD88 siRNA. The results indicate that GEF-H1, RhoA and MyD88 all not only promoted phosphorylation of NF-κB (Fig 5A and 5B), but also increased DNA binding activity of NF-κB in endothelial cells (Fig 5C and 5D). Moreover, compared with individual transfection of GEF-H1, RhoA or MyD88 siRNA, co-transfection with either GEF-H1 and MyD88 or RhoA and MyD88 siRNAs presented a more effective inhibition of LPS-induced phosphorylation and DNA binding activity of NF-κB (Fig 5B and 5D). These

results demonstrate that GEF-H1-RhoA and MyD88 both play important roles in LPS-induced phosphorylation and DNA binding activity of NF-κB in endothelial cells.

In endothelial cells, IL-8 expression is tightly controlled by NF-κB and activator protein (AP)-1 (Munoz et al., 1996; Mukaida et al., 1998). To clarify the roles of GEF-H1 and RhoA on LPS-induced IL-8 expression in HUVECs, we designed an IL-8 primer and amplified the cDNA for 31 cycles from total mRNA by RT-PCR. The primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified for 17 cycles as an internal control. The IL-8 protein within culture supernatants was detected by ELISA. As a result, compared with the negative control, LPS significantly enhanced expression of IL-8 mRNA and protein. Compared with the positive control, LPS-induced expression of IL-8 mRNA and protein were inhibited not only by transfection of MyD88 siRNA but also by transfection of GEF-H1 or RhoA siRNA. Furthermore, co-inhibition of GEF-H1 and MyD88 expression more effectively blocked LPS-induced expression of IL-8 mRNA and protein than in isolation. Similarly, co-inhibition of RhoA and MyD88 expression could produce more inhibitory effects on LPS-induced expression of IL-8 mRNA and protein than their individual inhibition. These data indicate that GEF-H1-RhoA and MyD88 both play important roles in LPS-induced expression of IL-8 mRNA and protein. (Fig 5E and 5F)

3.4. GEF-H1-RhoA signalling regulates LPS-induced NF-κB transactivation and IL-8 expression through p38 MAPK (Mitogen Activated Protein Kinases)

RhoA-regulated signalling can be mediated by a variety of downstream effectors (Vojtek and Cooper, 1995). Exposure of endothelial cells to LPS resulted in activation of several MAPKs. Activation of p38 appears to be of specific importance (Saha et al., 2007).

To address whether p38 acted as a downstream effector of LPS-induced GEF-H1-RhoA signalling, p38 phosphorylation was assessed in cell cultures treated with GEF-H1 and RhoA-specific siRNAs. In Fig 3B and 3D, LPS triggered a substantial increase in phosphorylation of p38 when compared with LPS-untreated control, However, LPS-induced phosphorylation of p38 was significantly decreased not only by transfection with GEF-H1 or RhoA siRNA alone but also by pretreatment with TcdB-10463. Moreover, there was no significant difference in LPS-induced p38 phosphorylation between individual and co-transfection of GEF-H1 and RhoA siRNAs. These results demonstrate that GEF-H1-RhoA signalling regulates LPS-induced phosphorylation of p38. While TcdB-10463 could suppress RhoA, Cdc42, and Rac activation, combined pretreatment with TcdB-10463 and RhoA siRNA did not augment inhibition of p38 phosphorylation compared to RhoA siRNA alone.

Furthermore, expression and phosphorylation of p38 were effectively down-regulated by transfection of p38-specific siRNA. Inhibition of p38 expression significantly reduced phosphorylation and DNA binding activity of NF-κB in the nucleus. The data indicates that inhibition of p38 could cause the inactivation of NF-κB. Inhibition of RhoA, GEF-H1 and p38 expression all significantly blocked LPS-induced phosphorylation and DNA binding activity of NF-κB in the nucleus. However, co-transfection of RhoA and p38 siRNAs or GEF-H1 and p38 siRNAs produced equivalent inhibitory effects on LPS-induced activation of p38 and NF-κB as transfection alone with p38 siRNA suggesting that GEF-H1-RhoA signalling mediated LPS-induced transactivation of NF-κB requires p38 activation. (Fig 6A, 6B, 6C and 6D)

While LPS-induced expression of IL-8 mRNA and protein was suppressed in p38 siRNA transfected-cells, co-inhibition of GEF-H1 and p38 expression did not produce further inhibition of LPS-induced IL-8 mRNA and protein expression. Similarly, RhoA and p38 co-inhibition also failed to produce enhanced inhibitory effects on LPS-induced mRNA and protein expression of IL-8 compared to their individual effects. Thus, p38 plays a key role in the GEF-H1-RhoA signalling cascade originating from LPS-TLR4 activation and leading to NF-κB transcriptional activation in order to promote expression of IL-8 mRNA and protein. (Fig 6E and 6F)

4. Discussion

In the pathogenesis of sepsis, endothelial cells respond to LPS through TLR activation, promoting the release of pro-inflammatory cytokines such as IL-8, and monocyte chemoattractant protein-1, along with increased expression of adhesion molecules (Weighardt and Holzmann, 2007). IL-8 is chronically elevated in patients with sepsis, and acts as a highly sensitive and specific diagnostic marker of bacterial sepsis (Stryjewski et al., 2005). However, recent studies have demonstrated that TLR signalling could be regulated by Rho GTPase activities (Ruse and Knaus, 2006). The activation state of Rho GTPases are in turn controlled by GEFs and GTPase-activating proteins (GAPs) that activate and inactivate them, respectively (Hall and Etienne-Manneville, 2002). Both affinity-based pull down assays and a FRET (Fluorescent Resonance Energy Transfer)-based biosensor for activated RhoA revealed GEF-H1 possesses RhoA-specific enzymatic activity (Bokoch et al., 2007). In this study we focused on identifying GEF-H1-RhoA signalling components regulated by TLR4 ligands in endothelial cells. Our results demonstrate that LPS-induced NF-κB transactivation and IL-8 synthesis in human endothelial cells depended on the convergence of two signalling pathways, specifically, the MyD88-dependent pathway and the GEF-H1-RhoA signalling pathway.

GEF-H1 represents a novel member of the Dbl family of guanine nucleotide exchange factors (GEFs) (Ren et al., 1998). Subcellular localization analysis demonstrates that GEF-H1 is associated with microtubules (MTs), and that MT depolymerization leads to GEF-H1 activation, accompanied by a RhoA-dependent reorganization of the actin cytoskeleton (Krendel et al., 2002). However, LPS could induce depolymerization of microtubules as early as 1 min after LPS stimulation, and this lasted for at least 4 h (Isowa et al., 2000). In the present study, stimulation with LPS resulted in dose- and time-dependent up-regulation of TLR4 and GEF-H1 synthesis. TcdB-10463, an inhibitor of Rho proteins, dose-dependently blocked LPS-induced phosphorylation of NF-κB, which indicated that Rho proteins were involved in the process of LPS-induced phosphorylation of NF-κB. Inhibition of GEF-H1 or RhoA expression significantly reduced LPS-induced phosphorylation of NF-κB in the nucleus. Taken together, it seems reasonable that GEF-H1 and RhoA proteins are involved in LPS-induced NF-κB activation in endothelial cells.

Exposure of endothelial cells to LPS-TLR4 signalling can selectively activate the MyD88-dependent pathway and MyD88-independent pathway (Akira and Takeda, 2004). Previous studies have found TLR4-induced activation of Cdc42 and Rac appears to be independent of MyD88, and LPS-TLR4 complexes provoke phagocytosis of macrophages though Cdc42/Rac pathway (Kong L and Ge BX, 2008). In the study presented, inhibition of TLR4 expression not only significantly reduced LPS-induced RhoA activities, but also blocked LPS-induced transactivation of NF-κB. These data indicate that TLR4 plays a pivotal role in regulating LPS-induced activation of RhoA. However, inhibition of MyD88 expression did not significantly block LPS-

induced RhoA activities, although MyD88 knockout significantly suppressed LPS-induced transactivation of NF-κB. These data suggest that LPS interacts with TLR4 to activate GEF-H1, then promoting RhoA signalling to NF-κB phosphorylation through a MyD88-independent pathway. However, compared with inhibition of GEF-H1 or RhoA expression, not only co-inhibition of RhoA and MyD88 but also co-inhibition of GEF-H1 and MyD88 produced more inhibitory effects on LPS-induced NF-κB transactivation and IL-8 synthesis. Taken together, GEF-H1-RhoA signalling and MyD88 signalling both regulate LPS-induced NF-κB transactivation and IL-8 synthesis, however, MyD88 does not involve LPS-induced RhoA activation.

In numerous instances, p38 has been cited as an upstream NF-κB regulatory kinase. We further verified whether p38 activation is required in the LPS-induced GEF-H1-RhoA signalling cascade within endothelial cells. Our results have shown that inhibition of GEF-H1 or RhoA expression by specific siRNAs significantly reduced LPS-induced phosphorylation of p38, suggesting that LPS mediates activation of p38 in part through GEF-H1-RhoA signalling. However, pretreatment with TcdB-10463 did not enhance the inhibitory effect of RhoA siRNA on phosphorylation of p38, which implied that Rac and Cdc42 might play roles in the activation of NF-κB in a p38-independent manner.

However, activation of NF-κB is not contingent on p38 activity (Goebeler et al., 2001), which has been reported in previous studies (Saha et al., 2003; Rajan et al., 2008). For this reason, we need to demonstrate whether GEF-H1-RhoA-mediated phosphorylation of p38 could lead to NF-κB transactivation and IL-8 synthesis. As a result, suppression of p38 by specific siRNA has a direct inhibitory effect on LPS-induced NF-κB transactivation and IL-8 synthesis in the present study. Moreover, co-inhibition of RhoA and p38 expression did not produce an augmented inhibitory effect on LPS-induced NF-κB transactivation and IL-8 synthesis than inhibition of p38 expression alone. Inhibition of NF-κB transactivation and IL-8 synthesis was also observed in endothelial cells co-transfected with GEF-H1 and p38 siRNAs. These data indicated that p38 as a downstream effector was essential for GEF-H1-RhoA-mediated NF-κB transactivation and IL-8 synthesis after exposure to LPS.

5. Conclusions

The present study further illustrates the complicated LPS-induced signalling involved in NF-κB transactivation and IL-8 expression. On one hand, LPS increases p38 phosphorylation, NF-κB transactivation and IL-8 expression, steps that require functionally activate GEF-H1 and RhoA and MyD88. While on the other hand, MyD88 is not involved in LPS-induced GEF-H1-RhoA signalling to p38 activation. Taken together, the data presented here suggest the existence of at least two LPS-induced pathways in endothelial cells that lead to NF-κB-dependent IL-8 expression through p38 phosphorylation: MyD88 signalling pathway and GEF-H1-RhoA signalling pathway.

Conflict of interest statement

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgements

This work was supported by the Chinese National Natural Science Foundation (No.81071553 and No. 30872686).

References

Aird W. C., 2003. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101, 3765-3777.

Ait-Oufella H., Maury E., Lehoux S., Guidet B., Offenstadt G., 2010. The endothelium: physiological functions and role in microcirculatory failure during severe sepsis. Intensive Care Med 36, 1286-1298.

Akira S., Takeda K., 2004. Toll-like receptor signalling. Nat Rev Immunol 4, 499-511.

Baggiolini M., Dewald B., Moser B., 1997. Human chemokines: an update. Annu Rev Immunol 15, 675-705.

Birkenfeld J., Nalbant P., Bohl B.P., Pertz O., Hahn K.M., Bokoch G.M., 2007. GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases. Developmental Cell 12 699-712.

Cook-Mills J.M., Deem T.L., 2005. Active participation of endothelial cells in inflammation. J Leukoc Biol 77, 487-495.

Doe C., Bentley R., Behm D.J., Lafferty R., Stavenger R., Jung D., Bamford M., Panchal T., Grygielko E., Wright L.L., Smith G.K., Chen Z., Webb C., Khandekar S., Yi T., Kirkpatrick R., Dul E., Jolivette L., Marino J.P. Jr, Willette R., Lee D., Hu E., 2007. Novel Rho kinase inhibitors with anti-inflammatory and vasodilatory activities. J Pharmacol Exp Ther 320, 89-98.

Fiorentini C., Falzano L., Travaglione S., Fabbri A., 2003. Hijacking Rho GTPases by protein toxins and apoptosis: molecular strategies of pathogenic bacteria. Cell Death Differ 10, 147-152.

Goebeler M., Gillitzer R., Kilian K., Utzel K., Bröcker E.B., Rapp U.R., Ludwig S., 2001. Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoatt Hijacking Rho GTPases by protein toxins and apoptosis: molecular strategies of pathogenic bacteria ractant protein-1 gene in primary endothelial cells. Blood 97, 46-55.

Hall, A., 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 10, 31-54.

Hall, A., S. Etienne-Manneville., 2002. Rho GTPases in cell biology. Nature 420, 629-635.

Hippenstiel S., Soeth S., Kellas B., Fuhrmann O., Seybold J., Krüll M., Eichel-Streiber C., Goebeler M., Ludwig S., Suttorp N., 2000. Rho proteins and the p38-MAPK pathway are important mediators for LPS-induced interleukin-8 expression in human endothelial cells. Blood 95, 3044-3051.

Isowa N., Keshavjee S.H., Liu M., 2000. Role of microtubules in LPS-induced macrophage inflammatory protein-2 production from rat pneumocytes. American Journal of Physiology-Lung Cellular and Molecular Physiology 279, L1075-L1082.

Kawai T., Adachi O., Ogawa T., Takeda K., Akira S., 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115-122.

Kim, J. H., C. H. Woo., 2002. Rac GTPase activity is essential for lipopolysaccharide signaling to extracellular signal-regulated kinase and p38 MAP kinase activation in rat-2 fibroblasts. Mol. Cells. 13, 470-475.

Kong L., Ge BX., 2008. MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis. Cell Res. 18, 745-755.

Krendel M., Zenke F.T., Bokoch G.M., 2002. Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. Nature Cell Human chemokines: an update. Annu Rev Immunol Biology 4, 294-301.

Liu-Bryan R., Pritzker K., Firestein G.S., Terkeltaub R., 2005. TLR2 signaling in chondrocytes drives calcium pyrophosphate dihydrate and monosodium urate crystal-induced nitric oxide generation. J. Immunol. 174, 5016-5023.

Mukaida N., Matsumoto T., Yokoi K., Harada A., Matsushima K., 1998. Inhibition of neutrophil-mediated acute inflammation injury by an antibody against interleukin-8 (IL-8). Inflamm. Res. 47, S151-157.

Muñoz C., Pascual-Salcedo D., Castellanos M.C., Alfranca A., Aragonés J., Vara A., Redondo J.M., de Landázuri M.O., 1996. Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity. Blood 88(9): 3482-3490.

Saha R.N., Jana M., Pahan K., 2007. MAPK p38 regulates transcriptional activity of NF-kappaB in primary human astrocytes via acetylation of p65. J. Immunol. 179, 7101-7109.

Saha R.N., Jana M., Pahan K., 2003. The p38 mitogen-activated protein kinase regulates interleukin-1beta-induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells. Immunology 108, 502-512.

Perona R., Montaner S., Saniger L., Sánchez-Pérez I., Bravo R., Lacal J.C., 1997. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. Genes. Dev. 11, 463-475.

Shibolet O., Giallourakis C., Rosenberg I., Mueller T., Xavier R.J., Podolsky D.K., 2007. AKAP13, a RhoA GTPase-specific guanine exchange factor, is a novel regulator of TLR2 signaling. J. Biol. Chem. 282, 35308-35317.

Rajan S., Ye J., Bai S., Huang F., Guo Y.L., 2008. NF-kappaB, but not p38 MAP kinase, is required for TNF-alpha-induced expression of cell adhesion molecules in endothelial cells. J Cell. Biochem. 105, 477-486.

Ren, X. D., M. A. Schwartz, 2000. Determination of GTP loading on Rho. Methods. Enzymol. 325, 264-272.

Ren Y., Li R., Zheng Y., Busch H., 1998. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. J. Biol. Chem. 273,34954-34960.

Riento, K., A. J. Ridley., 2003. Rocks: multifunctional kinases in cell behaviour. Nat. Rev. Mol. Cell. Biol. 4, 446-456.

Ruse M., Knaus U.G., 2006. New players in TLR-mediated innate immunity: PI3K and small Rho GTPases. Immunol. Res. 34, 33-48.

Smart S.J., Casale T.B., 1993. Interleukin-8-induced transcellular neutrophil migration is facilitated by endothelial and pulmonary epithelial cells. Am. J. Respir. Cell. Mol. Biol. 9, 489-495.

Stryjewski G.R., Nylen E.S., Bell M.J., Snider R.H., Becker K.L., Wu A., Lawlor C., Dalton H., 2005. Interleukin-6, interleukin-8, and a rapid and sensitive assay for calcitonin precursors for the determination of bacterial sepsis in febrile neutropenic children. Pediatr. Crit. Care. Med. 6, 129-135.

Vojtek, A. B., J. A. Cooper., 1995. Rho family members: activators of MAP kinase cascades. Cell. 82, 527-529.

Weighardt, H., B. Holzmann., 2007. Role of Toll-like receptor responses for sepsis pathogenesis. Immunobiology. 212, 715-722.

Wendel M., Paul R., Heller A.R., 2007. Lipoproteins in inflammation and sepsis. II. Clinical aspects. Intensive. Care. Med. 33, 25-35.

Wertheim W.A., Kunkel S.L., Standiford T.J., Burdick M.D., Becker F.S., Wilke C.A., Gilbert A.R., Strieter R.M., 1993. Regulation of neutrophil-derived IL-8: the role of prostaglandin E2, dexamethasone, and IL-4. J. Immunol. 151, 2166-2175.

Yasumoto K., Okamoto S., Mukaida N., Murakami S., Mai M., Matsushima K., 1992. Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. J. Biol. Chem. 267, 22506-2251.

Zheng, Y., 2001. Dbl family guanine nucleotide exchange factors. Trends. Bio. chem. Sci. 26, 724-732.

Figures

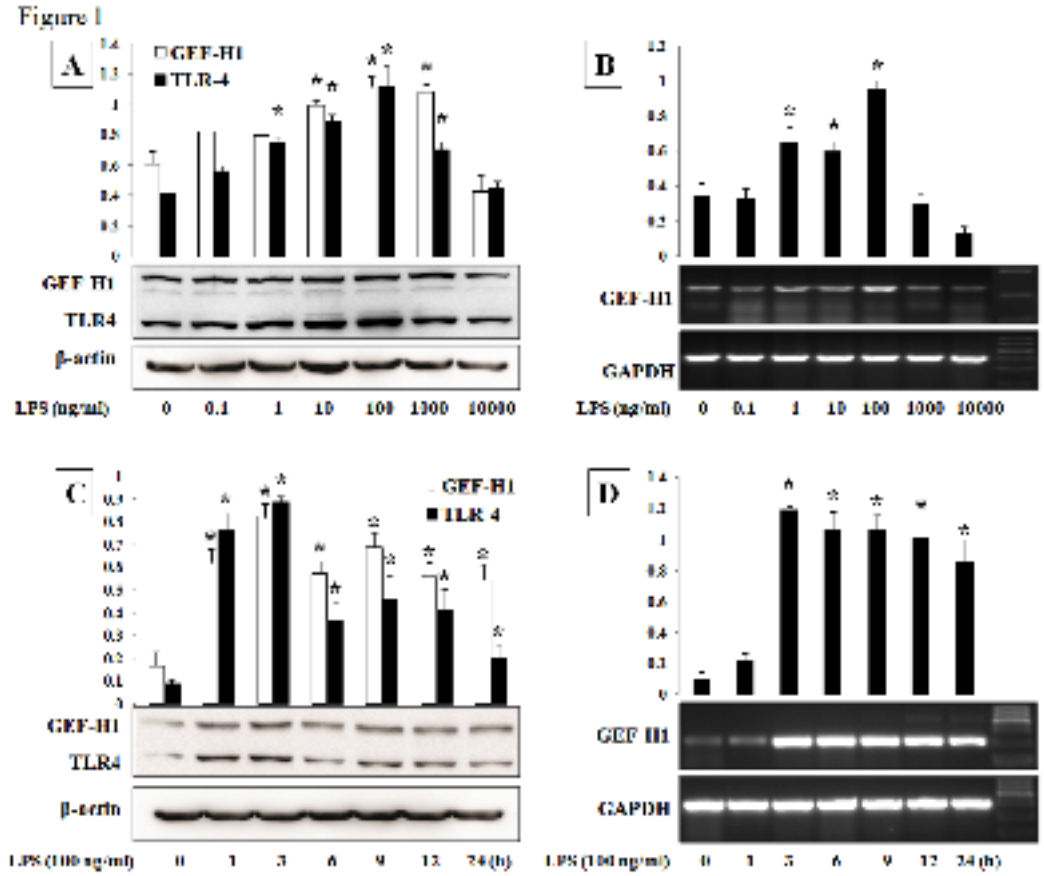

Fig. 1. LPS induced GEF-H1 and TLR4 expression in dose- and time-dependent manners in endothelial cells. (A) Expression of GEF-H1 and TLR4 after treatment with different concentrations of LPS in endothelial cells for 24 hours, and peak appeared at concentration of 100 ng/ml. **(B)** GEF-H1 mRNA expressed in a similar dose-dependent manner. **(C)** Expression of GEF-H1 and TLR4 in different indicated time points after stimulation of LPS with concentrations of 100 ng/ml in endothelial cells, and maximum expression appeared at 3 hours. **(D).** GEF-H1 mRNA expressed in a similar time-dependent manner. (* $P<0.05$ versus negative control). Data shown are representative of four independent experiments.

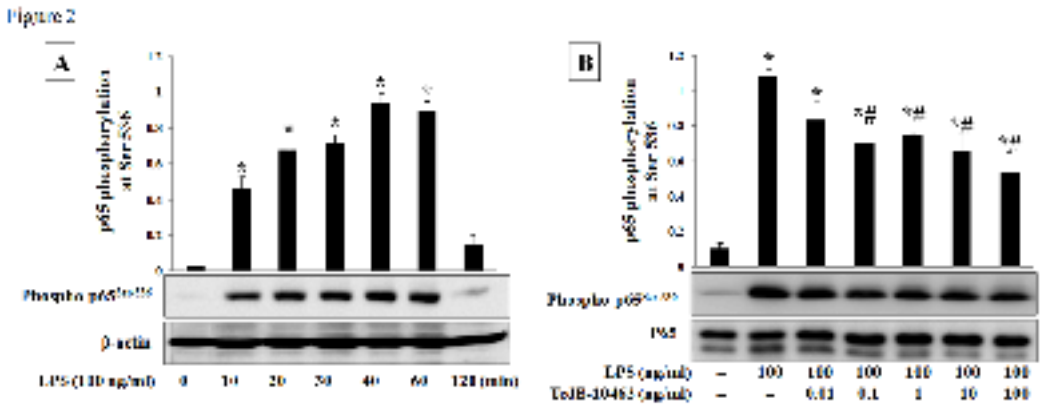

Fig. 2. Inhibition of Rho proteins blocks LPS-induced phosphorylation of NF-κB in endothelial cells. (A) HUVECs were exposed to 100 ng/ml LPS for the indicated times. LPS induced a time-dependent increase of phosphorylation of NF-κB at Ser-536 (phospho-p65$^{Ser-536}$) within 120 minutes in cytoplasmic extractions, and the maximum level of phospho-p65$^{Ser-536}$ appeared at 40 min after LPS stimulation. **(B)** Cells were preincubated with 0.01 to 100 ng/ml of TcdB-10463 (inhibitor of RhoA, Cdc42, and Rac) for 60 minutes prior to stimulation with 100 ng/ml LPS for 40 minutes. The TcdB-10463 inhibited LPS-related phospho-p65$^{Ser-536}$ in HUVECs in a dose-dependent manner. Treatment alone with medium or LPS was used as a negative control or positive control. (* P<0.05 versus negative control; # P<0.05 versus positive control). Data shown are representative of four independent experiments.

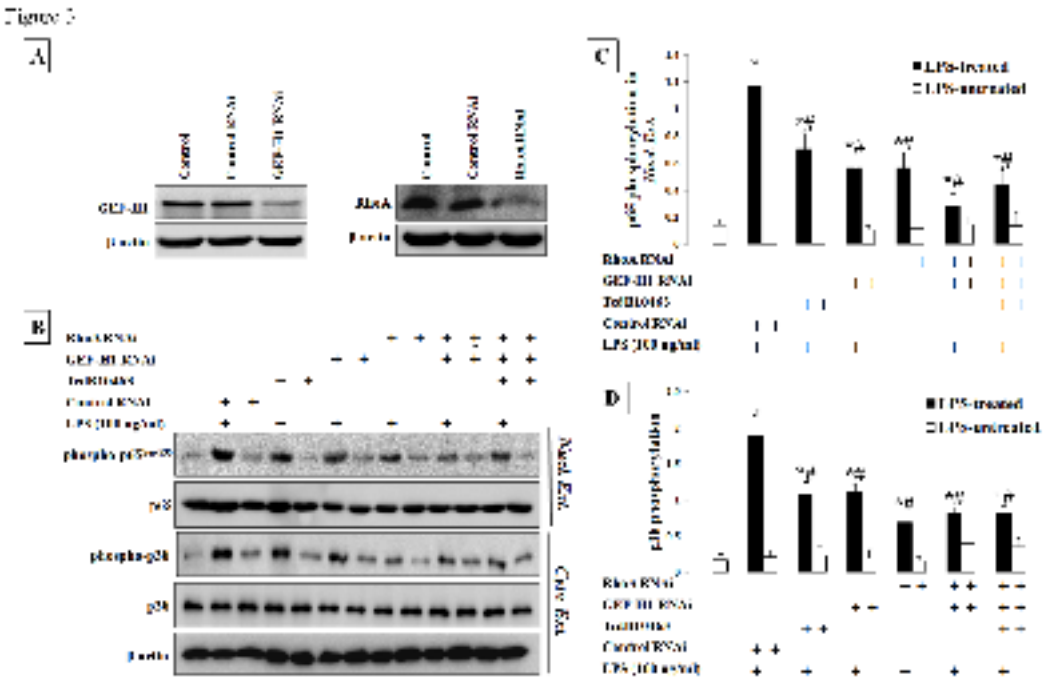

Fig. 3. GEF-H1-RhoA signalling is required for LPS-induced NF-κB and p38 phosphorylation. (A) HUVECs were transfected with specific siRNAs (RhoA, MyD88 or GEF-H1 siRNAs) or control siRNA for 48 h, and abilities of specific siRNAs were detected by western blot assays. **(B)** After transfection with specific siRNAs and control siRNA, HUVECs were treated with TcdB-10463 (10 ng/ml) for 60 min prior to LPS stimulation (100 ng/ml) for 40 minutes. Phosphorylation of p65 at Ser 536 was determined by immunoblotting of cell nuclear extracts (*Nucl. Ext.*), and β-actin protein was used as loading control. Cytosolic extracts (*Cyto. Ext.*) were immunoblotted with antibodies for phospho-p38 and p38 antibodies. β-actin protein was used as loading control. The levels of p65 and p38 phosphorylation were represented as a histogram according to band intensities as shown in **(C)** and **(D)**. After control siRNA transfection, the cells treated with medium or LPS were used as negative control or positive control respectively. (* P<0.05 versus negative control; # P<0.05 versus positive control). Data shown are representative of four independent experiments.

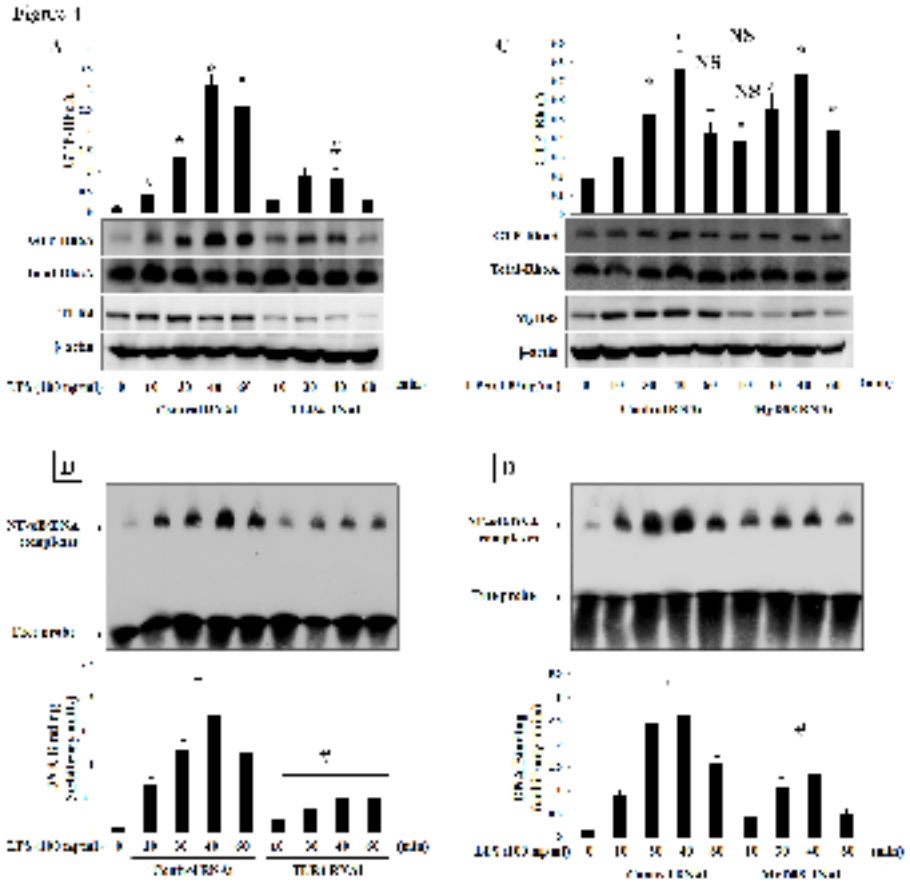

Fig. 4. TLR4 regulates LPS-induced RhoA activity and NF-κB transactivation. (A) HUVECs transfected with TLR4 siRNA were treated with medium or LPS (100 ng/ml) for indicated times, and GTPase RhoA activity assay was performed as described above. There was a significant difference in RhoA activity between control siRNA and TLR4 siRNA. **(B)** Inhibition of TLR4 expression significantly reduced NF-κB DNA binding activity. The results of EMSA were represented in a histogram. **(C)** Inhibition of MyD88 expression did not block LPS-induced GTPase RhoA activity. **(D)** NF-κB DNA binding activity was monitored by EMSA. Inhibition of MyD88 expression significantly reduced LPS-induced NF-κB activity. (* $p<0.05$ versus negative control; # $p<0.05$ versus corresponding control siRNA; NS, no significance). Data shown are representative of four independent experiments.

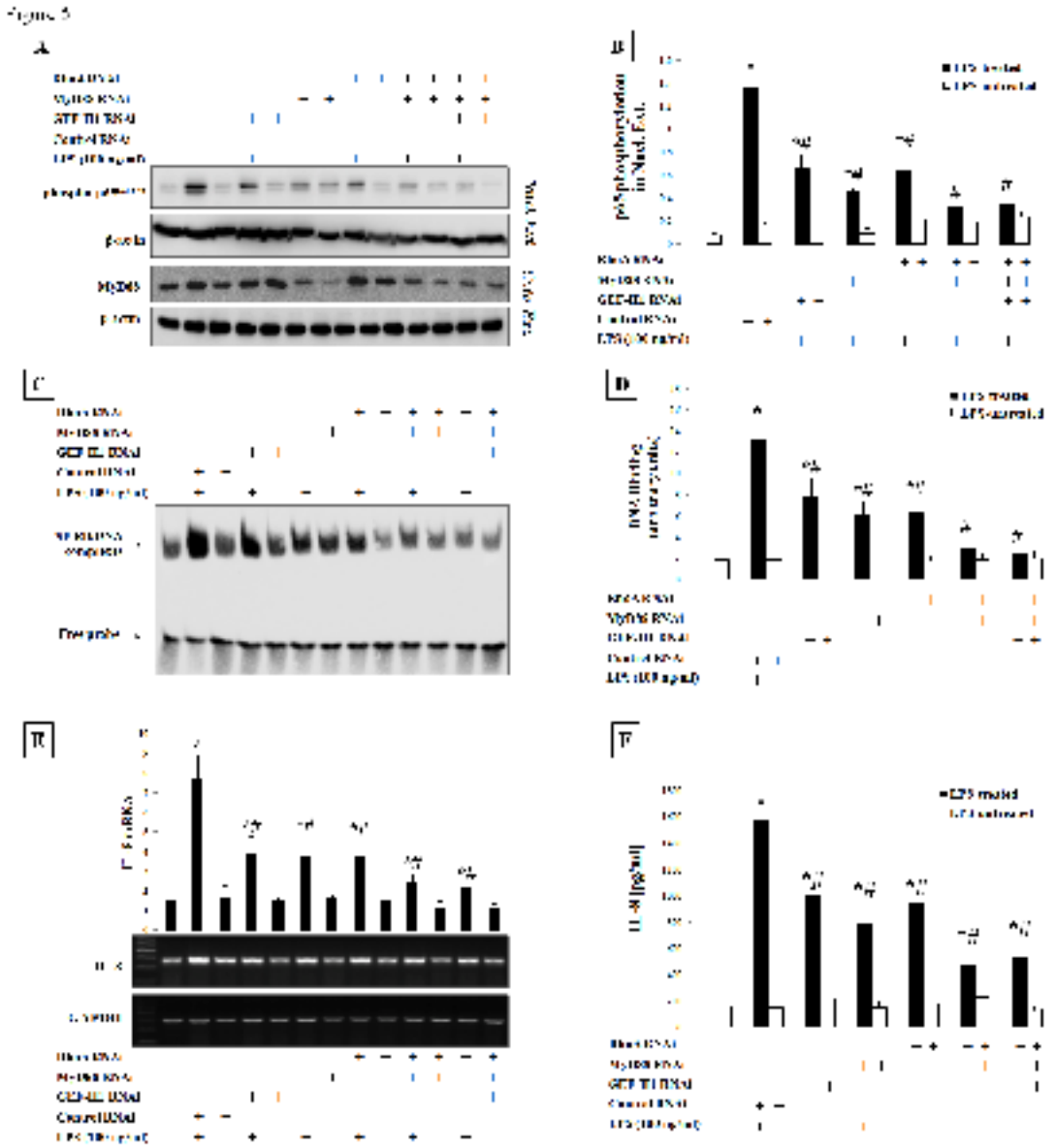

Fig. 5. **GEF-H1-RhoA and MyD88 both regulate LPS-induced NF-κB transactivation and IL-8 synthesis.** **(A)** HUVECs transfected with specific siRNAs (RhoA, MyD88 or GEF-H1 siRNAs) or negative control siRNA (control RNAi) or left untransfected (control) for 48 h, and ability of MyD88 siRNA was detected by Western blotting. After transfection, HUVECs were treated with media or LPS (100 ng/ml) for 40 minutes. Phosphorylation of p65 at Ser 536 (phospho-p65$^{Ser\text{-}536}$) was determined by immunoblotting in cell nuclear extracts (*Nucl. Ext.*), and the results were represented as a histogram in **(B)** according to band intensities. **(C)** Nuclear extracts were incubated with NF-κB-specific, biotinylated, double-stranded oligonucleotides, and detected by EMSA, and the results were represented as a histogram in **(D)** according to band intensities. **(E)** IL-8 mRNA was amplified for 31 cycles in total RNA by RT-PCR assay. GAPDH mRNA was amplified for 17 cycles as an internal control. **(F)** Secretion of IL-8 in supernatants was detected by ELISA. After control siRNA transfection, the cells treated with medium or LPS were used as negative control or positive control respectively. (*P<0.05 versus negative control; # P<0.05 versus positive control). Data shown are representative of four independent experiments.

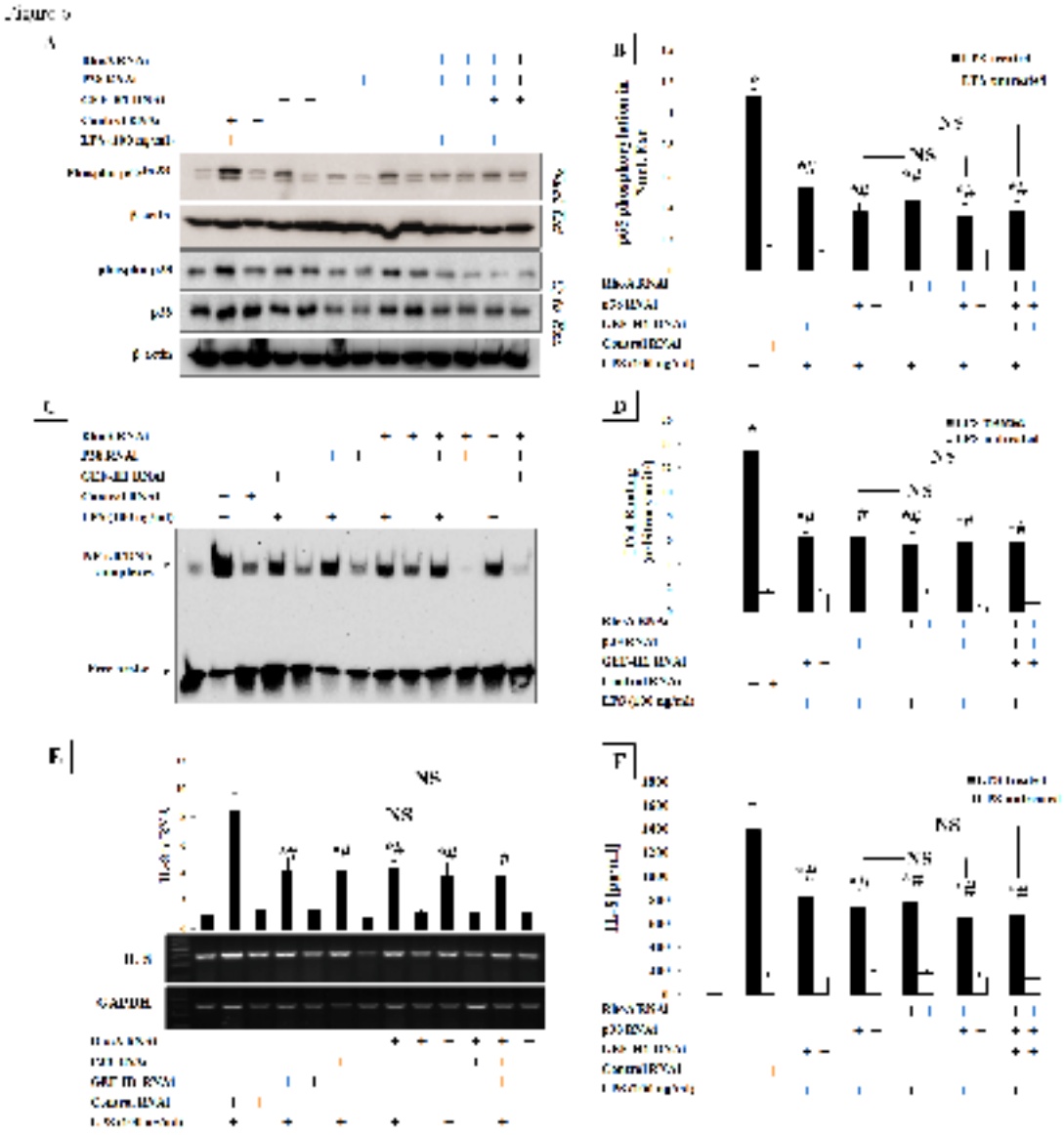

Fig. 6. GEF-H1-RhoA signalling regulates LPS-induced NF-κB phosphorylation through p38 activation. **(A)** HUVECs transfected with specific siRNAs (RhoA, p38 or GEF-H1 siRNA) or negative control siRNA (control RNAi) or left untransfected (control) for 48 h, and ability of p38 siRNA was detected by western blot assays. After transfection, HUVECs were treated with media or LPS (100 ng/ml) for 40 min. Phospho-p65$^{Ser-536}$ was determined by immunoblotting of cell nuclear extracts (*Nucl. Ext.*), and the levels of phospho-p65$^{Ser-536}$ were evaluated by the band intensities in **(B)**. **(C)** DNA binding activity of NF-κB was detected by EMSA, and the results were represented as a histogram in **(D)** according to band intensities**. (E)** IL-8 mRNA was examined by RT-PCR assay. GAPDH mRNA was used as an internal control. **(F)** Secretion of IL-8 in supernatants was detected by ELISA. After control siRNA transfection, the cells treated with medium or LPS were used as negative control or positive control respectively. (*P<0.05 versus negative control; # P<0.05 versus positive control; NS, no significance). Data shown are representative of four independent experiments.