LPS-induced nuclear translocation of RhoA is dependent on NF-κB in the human lung cancer cell line A549

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Abstract. RhoA, an extensively studied member of the Rho GTPase family, has been identified as a mediator of pro-inflammatory responses and aggressive carcinogenesis. Bacterial lipopolysaccharide (LPS) is known to be a potent stimulator of inflammatory cytokine production. LPS is able to alter the activity of RhoA and the subcellular distribution of RhoA is altered according to its activity. In this study, we investigated a possible link between RhoA and the LPS/nuclear factor (NF)-κB signaling pathway. In the present study, western blotting and pull-down and immunofluorescence assays were performed to investigate the activity of RhoA in A549 cells following LPS stimulation. The results showed that LPS was able to activate RhoA. Furthermore, western blotting and an immunofluorescence assay were carried out to investigate the nuclear expression of RhoA in A549 cells following LPS stimulation. The results indicated that LPS triggers the nuclear translocation of RhoA. Furthermore, western blotting, NF-κB small interfering RNA (siRNA) transfection and an immuno-fluorescence assay were performed to investigate the role of NF-κB in LPS-induced RhoA nuclear translocation in A549 cells. The results showed that LPS-induced RhoA nuclear translocation was inhibited by NF-κB depletion in A549 cells. RhoA and NF-κB siRNA transfection, western blotting and ELISA were carried out to investigate the role of RhoA in the LPS-induced secretion of interleukin (IL)-6 and IL-8 in A549 cells. The depletion of RhoA using RhoA siRNA decreased the LPS-induced secretion of IL-6 and IL-8, similar to the effect of NF-κB depletion. These results demonstrate that LPS is able to activate RhoA and trigger its nuclear translocation, which is dependent on NF-κB, and that RhoA plays a significant role in the LPS/NF-κB signaling pathway.

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Introduction

Previous studies have reported that the oncogene RhoA is involved in the regulation of a number of biological processes, including stress fiber formation, membrane transport, gene transcription, focal adhesion and tumor progression (1-4). RhoA is a small G protein and is therefore inactive when GDP-bound and active when GTP-bound, with GDP/GTP exchange or GTPase reactions converting one form to the other (5). The results of previous studies have shown that lyso-phosphatidic acid (LPA) is able to activate RhoA and induce its nuclear translocation and that the subcellular distribution of RhoA is correlated with its activity (6). Previous studies have reported that RhoA plays a significant role in CNS injuries and is a potential target of non-steroidal anti-inflammatory drugs (NSAIDs) in treating CNS injuries (7). This finding suggests a role of RhoA as an anti-inflammatory factor.

Bacterial lipopolysaccharide (LPS) is found in the outer membrane of Gram-negative bacteria and is able to activate a number of mammalian cell types and intracellular signaling pathways. For instance, it has been reported that LPS activates nuclear factor (NF)-κB, inducing the production and release of numerous pro-inflammatory mediators, including inter-leukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α. The synthesis and release of pro-inflammatory cytokines in response to LPS depends on inducible gene expression, which is mediated by the activation of transcription factors, including NF-κB. NF-κB regulates various genes involved in immune and acute phase inflammatory responses and in cell survival (8). Pro-inflammatory stimuli lead to the activation of NF-κB via the phosphorylation of inhibitors of κB (IκBs) by the IκB kinase (IKK) signalosome complex (9). This frees NF-κB and allows it to translocate to the nucleus of the cell where it induces the transcription of pro-inflammatory media-tors, including iNOS, COX-2, TNF-α, IL-1, IL-6 and IL-8, by binding to κB binding sites in the promoter regions of the target genes (10). However, the detailed molecular anti-inflammatory mechanism has not yet been studied. Since LPS is able to acti-vate NF-κB and trigger its nuclear translocation and alter the activity of RhoA, we suggested a link between RhoA and the LPS/NF-κB signaling pathway.

In the present study, we investigated the activity and nuclear distribution of RhoA following LPS stimulation and revealed for the first time that LPS triggers the nuclear translocation of RhoA, which is dependent on NF-κB. Furthermore, we

confirmed that RhoA is critical for the LPS/NF-κB signaling pathway in epithelial inflammation.

Materials and methods

Cell line. The human lung cancer cell line A549 was provided by the Institute of Cell Biology (Shanghai, China).

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Antibodies against RhoA (Cat. No. sc-418) and NF-κB P50 (Cat. No. sc-114) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). FITC, TRITC and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) and the Nuclear and Cytoplasmic Extract kit (Cat. No. KC-435) were purchased from Kangcheng Bio-tech (Hangzhou, China). RhoA small interfering RNA (siRNA) (Cat. No. sc-29471), NF-κB P50 siRNA (Cat. No. sc-29407) and transfection reagent (Cat. No. sc-29528) were purchased from Santa Cruz Biotechnology, Inc.. Nuclear fluorochrome Hoechst 33342 was purchased from Sigma (St. Louis, MO, USA), Electrochemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Buckinghamshire, UK).

Cell culture and transfections. A549 cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 IU/ml and 100 mg/ml, respectively) and incubated at 37°C and 5% CO_2. The cells were seeded in six-well plates at a density of 80% confluence and transfected the following day. The transfection of siRNA was performed according to the manufacturer's instructions.

Preparation of cell extracts. The cells were harvested at various times by aspiration of the media and direct addition of 2X SDS sample buffer. The cell lysate was scraped and transferred to tubes, heated for 5 min at 95°C and stored at -20°C.

Nucleus and cytosol preparation. The cells were seeded in six-well plates at a density of 80% confluence and treated with or without 10 μg/ml LPS for 4 h. The nuclear extracts were prepared according to the instructions of the manufacturer of the Nuclear and Cytoplasmic Extract kit.

RhoA activation assay. RhoA activity was determined as described in a previous study (11). This pull-down assay uses the RhoA-binding domain (RBD) from the effector protein Rhotekin as a probe to specifically isolate the active forms of RhoA. Briefly, A549 cells were cultured in 100 mm culture dishes with complete medium (DMEM with 10% FBS and antibiotics) until 90% confluence was achieved. Following 24 h of starvation, the cells were stimulated with 10 μg/ml LPS for 5 min and then lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 2 μg/ml each of leupeptin and aprotinin, 1 μl/ml phenylmethylsulphonyl fluoride and 0.5 μl/ml DTT). The cellular proteins were harvested by scraping with a rubber policeman. The lysates were centrifuged at 4°C at 14,000 x g for 5 min to remove particulate material and the protein concentrations were determined using the Bradford assay. The protein extracts (25 μg of each) were incubated while rotating at 4°C for 3 h with an equal volume of Rhotekin-RBD bound to glutathione-agarose beads. The beads were washed three times with washing buffer and activated RhoA bound to the beads or total RhoA in cell extracts was detected using western blot analysis with a monoclonal antibody against RhoA.

Western blot assay. The sample proteins were separated by SDS-PAGE and blotted onto a polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature. The membrane was incubated at 4°C overnight with the primary antibody and at room temperature for 1 h with the secondary antibody, with three washes following each incubation. ECL reagents were used to visualize the positive bands on the membrane. The bands were detected by Typhoon 9400 (GE Healthcare, Piscataway, NJ, USA).

Immunofluorescence assay. Forty-eight hours after transfection, control and NF-κB P50 siRNA-transfected cells grown on coverslips were treated with or without 10 μg/ml LPS for 4 h, incubated with 0.2 mM Hoechst 33342 for 10 min to reveal the nuclei and then fixed with freshly prepared 40 g/l paraformaldehyde in PBS at 4°C overnight. After being penetrated with 30 ml/l Triton X-100 and blocked with 30 g/l BSA, the cells were incubated with primary antibodies at 4°C overnight and then with cy3-conjugated second antibodies for 1 h at room temperature, with three washes following each incubation. The morphological changes of the cells were analyzed using a fluorescence microscope.

Determination of IL-8 and IL-6. Forty-eight hours after transfection, control, RhoA and NF-κB P50 siRNA-transfected A549 cells were treated with or without LPS for 24 h. The supernatants were then harvested and assessed for IL-8 and IL-6 production by ELISA (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. Values are shown as the mean ± SE (n=6). The Student's t-test was used for comparisons of two sample means. $P<0.05$ was considered to indicate a statistically significant result.

Results

LPS activates RhoA and triggers its nuclear translocation in A549 human lung cancer cells. Pro-inflammatory cytokines and bacterial endotoxins activate the Rho GTPase signaling pathway, which mediates the pro-inflammatory responses and aggressive carcinogenesis (12-14). To determine whether LPS was able to activate RhoA in A549 cells, we performed a pull-down assay to detect the activity of RhoA following LPS stimulation. The A549 cells were harvested and the active RhoA in the extract was isolated using the pull-down assay following treatment with 10 μg/ml LPS for 10 min and analyzed by western blotting with an antibody against RhoA. The results revealed that the level of active RhoA increased following LPS treatment (Fig. 1A). As there was a correlation between RhoA activity and its subcellular distribution, we determined the level
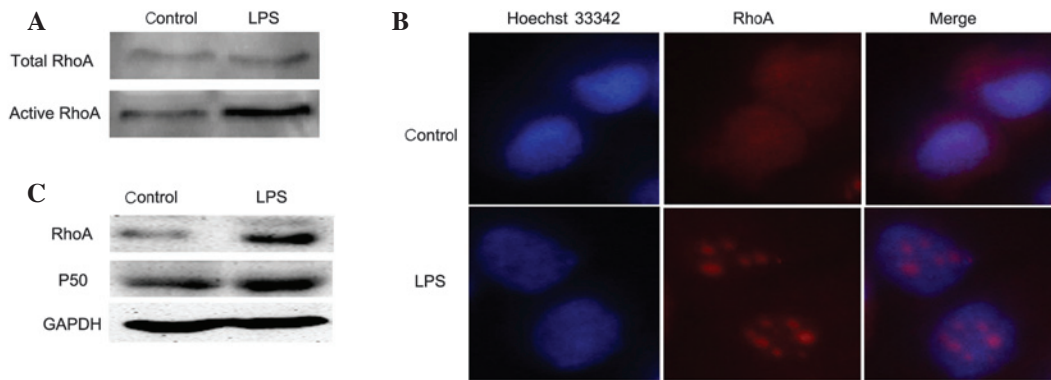
Figure 1. Expression of RhoA induced by LPS in the nuclei of A549 cells. A549 cells were treated with or without 10 μg/ml LPS for 4 h. (A) The activity of RhoA was analyzed using the pull-down assay. (B) The cells were stained with antibodies against RhoA (red) and with nuclear dye Hoechst 33342 (blue) to visualize the expression of RhoA in the nuclei. (C) The nuclear extracts were prepared and analyzed by western blotting with antibodies against RhoA and GAPDH. GAPDH expression served as a control. LPS, lipopolysaccharide.
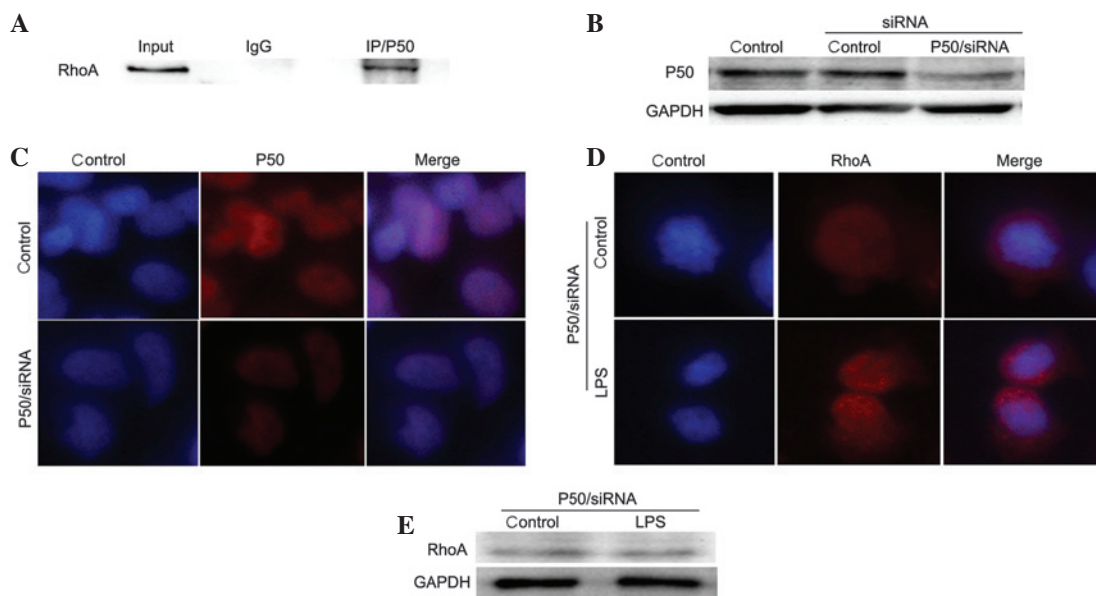

Figure 2. Expression of RhoA induced by LPS following NF-κB depletion in the nuclei. (A) The binding between NF-κB P50 and RhoA was detected using the Co-IP assay. (B) A549 cells were transfected with control siRNA and NF-κB P50 siRNA and the cell extracts were analyzed by western blotting with antibodies against NF-κB P50 and GAPDH at 48 h after transfection. (C) A549 cells were transfected with control siRNA and NF-κB P50 siRNA and stained with antibody against NF-κB P50 (red) and the nuclear dye Hoechst 33342 (blue) to visualize the expression of NF-κB P50. (D) Forty-eight hours after siRNA transfection, the cells were treated with 10 μg/ml LPS for 4 h, then stained with antibody against RhoA (red) and the nuclear dye Hoechst 33342 (blue) to show the expression of RhoA in the nuclei. (E) The cells were treated with 10 μg/ml LPS for 4 h and the nuclear extracts were prepared and analyzed by western blotting with antibodies against RhoA and GAPDH. GAPDH expression served as a control. LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; Co-IP, co-immunoprecipitation; siRNA, small interfering RNA.

of expression of RhoA in the nucleus following LPS stimulation. A549 cells were treated with or without 10 μg/ml LPS for 4 h. An immunofluorescence assay was performed to show the level of expression of RhoA in the nucleus with an antibody against RhoA (Fig. 1B). The nuclear extracts were then prepared following treatment with 10 μg/ml LPS for 4 h and analyzed by western blotting with antibodies against RhoA, NF-κB P50 and GAPDH (Fig. 1C). The results showed that LPS triggered the nuclear translocation of RhoA while increasing its activity, similar to the effect of LPS on NF-κB.

LPS-induced RhoA nuclear translocation is dependent on NF-κB. As LPS was able to trigger the nuclear translocation of both RhoA and NF-κB, we investigated whether the

LPS-induced RhoA nuclear translocation was dependent on NF-κB P50, since NF-κB, and not RhoA, contains nuclear localization signals (NLS). Firstly, we investigated if there was binding between RhoA and NF-κB. A co-immunoprecipitation (Co-IP) assay was performed with an antibody against NF-κB P50 to reveal any interaction. The results showed that there was an association between RhoA and NF-κB (Fig. 2A). Secondly, we detected the effect of NF-κB P50 depletion on LPS-induced RhoA nuclear translocation. We used NF-κB P50 siRNA to deplete NF-κB P50 in A549 cells. The effect of the NF-κB P50 siRNA on the expression of NF-κB was detected by western blotting and an immunofluorescence assay using an antibody against NF-κB P50. The results of the western blotting (Fig. 2B) and immunofluorescence assay
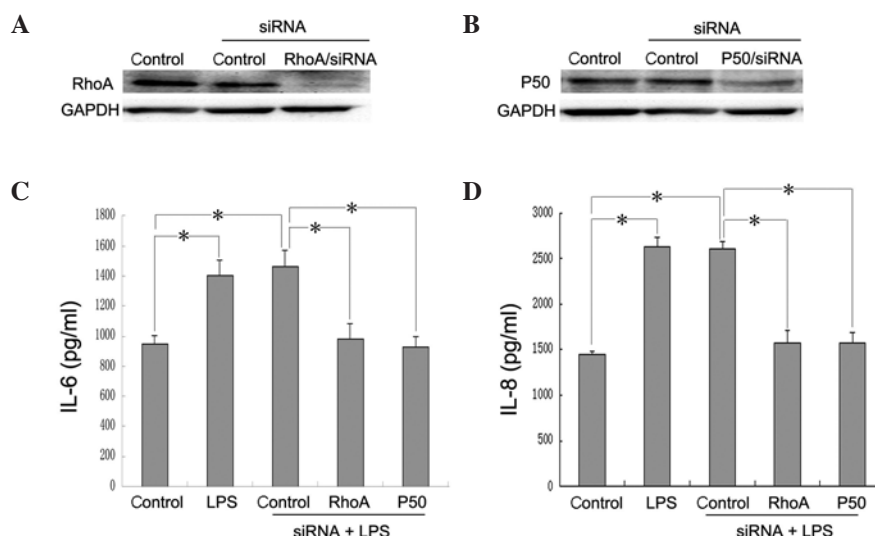
Figure 3. Effect of RhoA depletion on LPS-induced IL-6 and IL-8 secretion in A549 cells. (A and B) siRNAs of RhoA and NF-κB P50 were applied to deplete their expression in A549 cells. The effects of the siRNAs on their expression were detected by western blotting with antibodies against RhoA, NF-κB P50 and GAPDH. GAPDH expression served as a control. (C and D) Forty-eight hours after siRNA transfection, the cells were treated with or without 10 µg/ml LPS for 24 h and the concentration of IL-6 and IL-8 in the supernatants was measured by ELISA. Data are shown as the mean + SE (n=6). *P<0.05. LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; siRNA, small interfering RNA; IL, interleukin.

(Fig. 2C) revealed that the expression of NF-κB P50 decreased markedly. Cells with NF-κB P50 depletion were treated with or without 10 µg/ml LPS for 4 h and western blotting and an immunofluorescence assay with an antibody against RhoA were performed to detect the nuclear expression of RhoA. The results of the immunofluorescence assay (Fig. 2D) and western blotting (Fig. 2E) revealed that the nuclear expression of RhoA did not increase following LPS stimulation in NF-κB P50-depleted cells. These data demonstrate that LPS-induced nuclear translocation of RhoA was dependent on NF-κB.

Depletion of RhoA inhibits LPS-induced IL-6 and IL-8 secretion in A549 human lung cancer cells. Since LPS had a similar effect on the activity and nuclear distribution of RhoA and NF-κB and active NF-κB was able to mediate the transcriptional activation of pro-inflammatory genes, including IL-6 and IL-8, we investigated whether RhoA was involved in the LPS/NF-κB pathway. We applied the NF-κB P50 and RhoA siRNAs to deplete the expression of NF-κB P50 and RhoA in A549 cells, respectively. The effect of the RhoA and NF-κB P50 siRNAs on the expression of RhoA (Fig. 3A) and NF-κB P50 (Fig. 3B) were detected by western blotting with antibodies against RhoA, NF-κB P50 and GAPDH. Forty-eight hours after siRNA transfection, the cells were treated with 10 µg/ml LPS for 24 h and the concentration of IL-6 and IL-8 in the supernatants was measured by ELISA. The results showed that the depletion of RhoA inhibited the LPS-induced release of IL-6 (Fig. 3C) and IL-8 (Fig. 3D), similar to the effect of NF-κB depletion.

Discussion

The ability of Rho GTPases to function in signaling pathways has been reported to depend partly on the location of the proteins within the cell (15). However, the activity of GTPases is also regulated by three types of proteins: guanine nucleo-

tide exchange factors (GEFs) catalyze the exchange of GDP for GTP and thereby activate Rho (16); GTPase activating proteins (GAPs) enhance the hydrolytic ability of GTPases thereby inactivating the proteins (17); and guanine nucleotide dissociation inhibitors (GDIs) also inactivate the GTPases by removing them from the plasma membrane (PM) and maintaining them in an inactive form in the cytosol (18). The majority of Rho-GEFs are localized to the cytoplasm or the PM (16), but two GEFs, Net1 and Ect2, have been reported to localize to the nucleus at a steady state (19,20). These two GEFs contain an NLS that is necessary for the proteins to be targeted to the nucleus (21,22). As RhoA has no NLS, it was of note for us to reveal how the nuclear translocation occurred. In the present study, following treatment with LPS, the nuclear translocation of RhoA occurred and its activity increased. Since results of previous studies showed that LPS is able to activate NF-κB and induce its nuclear translocation, and as NF-κB contains an NLS, we hypothesized that the LPS-induced nuclear translocation of RhoA was mediated by NF-κB. The results of the present study show that the depletion of NF-κB by siRNA inhibited the LPS-induced nuclear translocation of RhoA. These data demonstrate that LPS-induced RhoA nuclear translocation is dependent on NF-κB.

LPS is a major inflammatory molecule that triggers the production of pro-inflammatory toxins and cytokines, including iNOS, COX-2, TNF-α and IL-1, in various cell types (23). As LPS activates RhoA and induces its nuclear translocation, it was important for us to investigate whether RhoA is involved in LPS-induced inflammatory cytokine secretion. In the present study, we have demonstrated that the depletion of RhoA by siRNA inhibits the LPS-induced secretion of IL-6 and IL-8.

In conclusion, we have shown a significant link between RhoA and the LPS/NF-κB signaling pathway. LPS activates RhoA and triggers its nuclear translocation, which is dependent on NF-κB; RhoA plays a significant role during LPS-induced

inflammatory cytokine secretion and the depletion of RhoA markedly inhibits the LPS-induced secretion of IL-6 and IL-8, similar to the effect of NF-κB depletion.

Acknowledgements

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