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Lipopolysaccharide stimulates syntheses of toll-like receptor 2 and surfactant protein-A in human alveolar epithelial A549 cells through upregulating phosphorylation of MEK1 and ERK1/2 and sequential activation of NF-κB

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ABSTRACT

Surfactant proteins (SPs) and toll-like receptors (TLRs) contribute to regulation of sepsis-induced acute lung injury. Lipopolysaccharide (LPS) is one of the major causes of septic shock. This study was designed to evaluate the effects of LPS on the regulation of *tlr-2* and *sp-a* gene expression in human alveolar epithelial A549 cells and the possible mechanisms. Exposure of A549 cells to LPS increased the expressions of TLR2 and SP-A mRNA and protein in time-dependent manners. A search using a bioinformatic approach found that there are several nuclear factor kappa-B (NF-κB)-DNA-binding motifs in the promoter region of the *tlr2* and *sp-a* genes. Immunoblotting analyses revealed that exposure to LPS time-dependently enhanced the translocation of NF-κB from the cytoplasm to nuclei. Analyses of an electrophoretic mobility shift assay further showed that LPS augmented the transactivation activity of NF-κB to its consensus oligonucleotides in A549cells. Sequentially, treatment of A549 cells with LPS increased phosphorylation of extracellular signal-regulated kinase (ERK)1/2, p38-mitogen-activated protein kinase (p38MAPK), and MAPK kinase-1 (MEK1). Pretreatment with PD98059, an inhibitor of ERK1/2, significantly decreased LPS-induced TLR2 and SP-A mRNA expression.

1. Introduction

Acute pulmonary infection culminating in acute respiratory distress syndrome is a critical complication in gram-negative bacterium-induced sepsis [1]. Lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, is one of the major causes of septic shock [2]. In the human lung, LPS-induced acute pulmonary inflammation causes rapid changes to the composition of the surfactant pool and resident cell population [3]. Functionally, pulmonary surfactant proteins (SPs) participate in regulating sepsis-induced acute lung injury. During inflammation, overproduction of SPs induced by a variety of drugs or endotoxins can cause alteration of pulmonary surfactant components and further affect the physical and pathophysiological functioning of the lung [4]. In response to immune stimulation, alveolar epithelial type II cells can synthesize SPs which maintain the mucosal integrity [5]. There are many types of SPs. In particular, SP-A, the most abundant pulmonary SP, can bind to LPS, viruses, fungal cell wall components, and pollen [6,7]. A previous study reported that a deficiency of SP-A increased lung susceptibility to pathogen infection [4]. Thus, alteration of lung SP-A levels can be an effective indicator of pulmonary infection and inflammation.

Toll-like receptors (TLRs) are type I transmembrane proteins with extracellular leucine-rich domains and intracellular signaling domains [8]. TLR2, a critical member of TLRs that exists in human pulmonary epithelial cells, participates in the response to lung injury [9,10]. In alveolar epithelial cells, LPS can specifically activate TLR2, which then triggers a cascade activation of mitogen-activated protein kinase (MAPK) family proteins and transcriptional factors [11]. In response to stimulation by LPS, nuclear factor-kap-

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pa B (NF-κB) and activator protein (AP)-1, two typical transcription factors that contribute to the mediation of TLR2-triggered signals, are upregulated [12]. Thus, the expression of TLR2 in cells plays crucial roles in immunoresponses. Also, SP-A can regulate surfactant homeostasis and function in the lung [13]. Our previous study showed that TLR2 is involved in regulating *sp-a* gene expression in human alveolar epithelial cells [14]. Meanwhile, the mechanisms of LPS-induced *tlr2* and *sp-a* gene expression in alveolar epithelial cells are still unknown. Therefore, in this study, we evaluated the effects of LPS on the regulation of *tlr2* and *sp-a* gene expression in human alveolar epithelial cells and the possible molecular mechanisms.

2. Materials and methods

2.1. Cell culture and drug treatment

Human lung carcinoma type II epithelium-like A549 cells, purchased from the American Type Culture Collection (Rockville, MD, USA), were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 culture medium (Sigma Chemical, St. Louis, MO, USA) with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 2 mM L-glutamine, and 100 μg/ml streptomycin in 75-cm^2 culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. LPS, purchased from Sigma, is extracted from *Escherichia coli* serotype O26:B6, and its product number is L3755. LPS was dissolved in dimethyl sulfoxide (DMSO) and sonicated to disperse large LPS aggregates. The concentration of DMSO in the medium was kept to <0.1% to avoid toxicity of this solvent to A549 cells. PD98059, an inhibitor of ERK1/2 [15], was purchased from Sigma, dissolved in DMSO, and pretreated at 10 μM for 1 h before LPS administration. Under such a treated condition, PD98059 did not cause cytotoxicity to A549 cells. Control cells received DMSO only.

2.2. Toxicity of LPS to A549 cells

Toxicity of LPS to A549 cells was assayed by analyzing cell morphologies and viability. Briefly, A549 cells (1×10^4 cells per well) were seeded overnight in 96-well tissue culture plates. After drug treatment, cell morphologies were observed and photographed using a reverse-phase light microscope. Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [16]. Following LPS treatment, A549 cells were cultured in new medium containing 0.5 mg/ml MTT for a further 3 h. The blue formazan products in A549 cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

2.3. Immunoblotting analyses of TLR2, SP-A, and β-actin

Protein analyses were carried out according to a previously described method [17]. After drug treatment, cell lysates were prepared in ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl (pH 7.2), 0.1% sodium dodecylsulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM EDTA). To avoid the degradation of the cytosolic proteins by proteinases, a mixture of 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate, and 5 μg/ml leupeptin was added to the RIPA buffer. Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce, Rockford, IL, USA). Proteins (50 μg/well) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. Immunodetection of TLR2 and SP-A was carried out using goat and rabbit polyclonal antibodies against human TLR2 and SP-A, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cellular β-actin protein was immunodetected using a mouse monoclonal antibody against mouse β-actin (Sigma) as the internal standard. These protein bands were quantified with the aid of UVI-DOCMW version 99.03 digital imaging system (UVtec, Cambridge, UK).

2.4. Reverse-transcription polymerase chain reaction (RT-PCR) assays of TLR2, SP-A, and β2M messenger (m)RNA

mRNA from A549 cells exposed to LPS was prepared for the RT-PCR analyses of TLR2, SP-A, and β2M mRNA as described previously [18]. Oligonucleotides for these PCR analyses were designed and synthesized by Clontech Laboratories (Palo Alto, CA, USA). The oligonucleotide sequences of the respective upstream and downstream primers for these mRNA analyses were 5′-TGAAAGGGAGTTC TAGCATCTCACAGA-3′ and 5′-ACATATGCCTATGTAGGCCTGACT-GAG-3′ for SP-A, 5′-GCCAAAGTCTTGATTGATTGG-3′ and 5′-TTGA AGTTCTTCAGCTCCTG-3′ for TLR2, and 5′-GTCTACATGTCTCGATCC-CACTTAA-3′ and 5′-GGTCTTTCTCTCTCATCGCGCTC-3′ for β2M. The PCR was carried out using 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min. The PCR products were loaded onto a 1.8% agarose gel containing 0.1 μg/ml ethidium bromide and electrophoretically separated. DNA bands were visualized and photographed under UV-light exposure. The intensities of the DNA bands in the agarose gel were quantified using a digital imaging system (UVtec).

2.5. Extraction of nuclear proteins and immunodetection

Nuclear components were extracted and immunodetected following the method of Chiu et al. [19]. After drug treatment, cytosolic and nuclear extracts of A549 cells were prepared. Protein concentrations were quantified with a bicinchonic acid protein assay kit (Pierce). Cytosolic and nuclear proteins (50 μg/well) were subjected to SDS–PAGE, and transferred to nitrocellulose membranes. After blocking, cytosolic and nuclear NF-κB was immunodetected using a rabbit polyclonal antibody against mouse NF-κB (Santa Cruz Biotechnology). Levels of total NF-κB were immunodetected as the internal standard. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

2.6. Electrophoretic mobility shift assay (EMSA)

An EMSA was performed using a Dig gel shift kit (Roche Diagnostics, Mannheim, Germany) as described previously [20]. Briefly, NF-κB consensus oligonucleotides (Santa Cruz Biotechnology) were labeled with Dig. The nuclear extracts (10 ng) were reacted with Dig-labeled oligonucleotides at room temperature for 25 min. The complex was subjected to non-denatured PAGE, and transferred to positively charged nylon membranes. After cross-linking at 120 mJ and blocking with the blocking buffer (Santa Cruz Biotechnology) at room temperature for 30 min, the membranes were immunoreacted with anti-Dig-AP. After one washing and chemiluminescent detection were completed, the membranes were exposed to X-ray film. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

2.7. Immunoblotting analyses of phosphorylated MEK1, ERK1/2, and p38MAPK

Protein analyses were carried out according to a previously described method [21]. After LPS treatment, cell lysates were prepared in ice-cold RIPA buffer. A mixture of 1 mM of phenyl methyl sulfonyl fluoride, 10 μg/ml of aprotinin, 1 mM of sodium orthovanadate, and 5 μg/ml of leupeptin was added to the RIPA buffer to avoid the degradation of cytosolic proteins by proteinase. Protein concentrations were quantified using a bicinchonic acid protein assay kit. Proteins