Lipopolysaccharide induces ICAM-1 expression via a c-Src/NADPH oxidase/ROS-dependent NF-κB pathway in human pulmonary alveolar epithelial cells

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Lung inflammation is a pivotal event in the pathogenesis of chronic obstructive pulmonary disease and asthma (13). These inflammatory responses are mediated by complex interactions between both circulating polymorphonuclear cells (PMNs) and the vascular endothelium. Several studies indicate that expression of adhesion molecules on the cell surface of endothelial cells plays a critical role in the inflammatory responses (13). Raised levels of adhesion molecules might contribute to the recruitment of PMNs to the regions of inflammatory tissue. These adhesion molecules are classified into two major families: the Ig superfamily [e.g., ICAM-1 and VCAM-1] and the selectins (e.g., P-selectin and E-selectin) (9). ICAM-1 is an inducible cell surface glycoprotein on several cell types, which mediates the tight adhesiveness of PMNs and thus facilitates PMN migration across the vascular endothelium barrier and then interacts with lung epithelium (8).

Lipopolysaccharide (LPS), a glycolipid component of the outer membrane of Gram-negative bacteria, exhibits the most potent immune-stimulating activity of the Toll-like receptor (TLR) ligands (20). Trace amounts of LPS are able to activate the innate immune system, leading to an array of proinflammatory mediators being produced, such as TNF-α and IL-1β. Therefore, LPS is generally acknowledged to play a central role, not only in eliciting inflammatory responses, but also in causing septic shock during Gram-negative bacterial infection (20). Recent studies have revealed that LPS recognition is complex and involves TLR4 (22). Moreover, LPS has been shown to regulate lung injury and inflammation (14, 32). In addition, LPS has been also shown to induce ICAM-1 or VCAM-1 expression in various cell types (15, 21, 30). However, the mechanisms of LPS-regulated ICAM-1 expression in human pulmonary alveolar epithelial cells (HPAEpiCs) are not completely understood. Thus, to clarify mechanisms of ICAM-1 induction by LPS in lung epithelium was recognized as a new therapeutic approach in the management of respiratory diseases.

Src family kinases are signaling enzymes that have long been recognized to regulate critical cellular processes, such as proliferation, survival, migration, and metastasis (3). c-Src has been shown to regulate ICAM-1 expression in various cell types (9, 16). In addition, LPS can mediate c-Src activation and then cause the inflammatory responses (7, 10). Previous studies indicated that c-Src regulates platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) transactivation (13), which further promotes inflammatory responses. NADPH oxidase is an enzymatic source for the production of reactive oxygen species (ROS) under various pathological conditions (13). The Src family kinases have been shown to regulate NADPH oxidase; lung inflammation; signaling pathway; adhesion molecules; transcription factor

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oxidase activation and ROS production (11). Thus, in this study, we investigated the roles of c-Src, NADPH oxidase/ROS, EGFR, and PDGFR in LPS-induced inflammatory responses, including ICAM-1 induction and monocyte adhesion.

Recent studies suggested that numerous components of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway play a crucial role in the expression and activation of inflammatory mediators, inflammatory cell recruitment, immune cell function, airway remodeling, and corticosteroid insensitivity in chronic inflammatory respiratory diseases (13). Indeed, previous studies indicated that PI3K/Akt regulates the expression of adhesion molecules in various cell types (16, 23, 25). LPS has been shown to enhance Akt activation (32). Moreover, p42/p44 MAPKs are related protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade. This cascade participates in the regulation of a large variety of cellular processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription (26).

Thus the key roles of PI3K/Akt and p42/p44 MAPK were studied in LPS-stimulated HPAEpiCs. NF-κB is a transcription factor involved in a wide variety of phenomena, including inflammation, immune responses, and cell survival. Abnormal activation of NF-κB occurs in many pathological conditions (5). LPS has been shown to stimulate NF-κB activation (27).

Therefore, LPS may play a potential role in regulation of expression of inflammatory genes, such as ICAM-1, and thereby promote inflammatory responses. We report here for the first time that LPS-induced ICAM-1 expression was mediated through a TLR4/c-Src/NADPH oxidase/ROS/PDGFR and EGFR/PI3K/Akt/p42/p44 MAPK/NF-κB-dependent pathway in HPAEpiCs.

MATERIALS AND METHODS

Materials. Anti-phospho-c-Src, anti-phospho-PDGFR, anti-phospho-EGFR, anti-phospho-Akt, anti-phospho-p65, anti-phospho-p42/p44 MAPK, anti-phospho-IκBα, anti-phospho-PKC-α, and anti-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**Fig. 1.** LPS induces ICAM-1 expression in human pulmonary alveolar epithelial cells (HPAEpiCs). **A:** cells were treated with various concentrations of LPS for the indicated time intervals. The protein levels of ICAM-1 were determined by Western blot. **B** and **C:** cells were treated with 10 μg/ml LPS for the indicated time intervals. The mRNA expression was determined by RT-PCR (B) or real-time PCR (C, open bars). The promoter activity of ICAM-1 was determined by a promoter assay (C, shaded bars). **D:** cells were incubated with LPS for 16 h, treated with an anti-ICAM-1-neutralizing antibody for 1 h, and then the THP-1 cell adhesion was measured. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05; #P < 0.01 compared with the cells exposed to vehicle alone (**A–C**). #P < 0.01 compared with the cells exposed to LPS alone (**D**).
Fig. 2. LPS induces ICAM-1 expression via c-Src in HPAEpiCs. A: cells were pretreated with protein phosphatase-1 (PP1) for 1 h and then incubated with LPS for 16 h. The levels of ICAM-1 protein were determined by Western blot. B: cells were transfected with either scrambled or c-Src siRNA and then incubated with LPS for 16 h. The levels of c-Src and ICAM-1 protein were determined by Western blot. C: cells were pretreated with PP1 for 1 h and then incubated with LPS for 4 h (mRNA levels) or 6 h (promoter activity). The mRNA levels of ICAM-1 were determined by real-time PCR (open bars). The promoter activity of ICAM-1 was determined by promoter assay (shaded bars). D: cells were pretreated with PP1 for 1 h, incubated with LPS for 16 h, and then measured for THP-1 cell adherence. E: cells were pretreated with PP1 for 1 h or transfected with c-Src siRNA and then incubated with LPS for the indicated time intervals. The levels of phospho-c-Src were determined by Western blot. F: cells were treated with LPS for the indicated time intervals. G: cells were transfected with scrambled, c-Src, or TNF receptor-associated factor 6 (TRAF6) siRNA and then incubated with LPS for 5 min. F and G: cell lysates were subjected to immunoprecipitation (IP) using an anti-c-Src, anti-TRAF6 or anti-MyD88 antibody. The immunoprecipitates were analyzed by Western blot using an anti-Toll-like receptor 4 (TLR4), anti-MyD88, anti-TRAF6, or anti-IgG antibody. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05; #P < 0.01 compared with the cells exposed to LPS alone. IB, immunoblots.
phospho-PKC-δ antibodies were from Cell Signaling Technology (Danvers, MA). Anti-ICAM-1, anti-c-Src, anti-GAPDH, anti-lamin A, anti-p65, anti-IκBα, anti-TLR4, anti-MyD88, anti-TNF receptor-associated factor 6 (TRAF6), anti-Rac1, anti-p47phox, anti-EGFR, anti-PDGFR, anti-Akt, anti-Nox2, anti-Nox4, anti-p42, anti-p44, anti-PKC-α, and anti-PKC-δ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein phosphatase-1 (PP1), AG1296, AG1478, diphenylenciondium chloride...
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(DPI), U0126, LY294002, H-7, and Bay11-7082 were from Biomol (Plymouth Meeting, PA). LPS (L2630), enyzmes, and other chemicals were from Sigma (St. Louis, MO). Edaravone was from Tocris Bioscience (Brussels, UK). Apocynin (APO) was purchased from ChromaDex (Santa Ana, CA). BCECF/AM, dihydroethidium (DHE), and CM-H2DCFH-DA were from Molecular Probes (Eugene, OR).

Cell culture. Human pulmonary alveolar epithelial cells (HPAEpiCs) were purchased from the ScienCell Research Laboratories (San Diego, CA) and grown as previously described (8). In this study, we observed the effects of various concentrations of LPS (0.1, 1.0, 10.0, or 50.0 μg/ml) on ICAM-1 expression. Moreover, the selected dose of 10 μg/ml LPS was used to investigate the signaling pathways involved in ICAM-1 expression.

Western blot analysis. HPAEpiCs were grown to confluence in six-well plates and then treated with 10 μg/ml LPS for the indicated time intervals. The cells were washed, scraped, collected, and centrifuged at 45,000 g at 4°C for 1 h to yield the whole cell extract, as previously described (8). Samples were denatured, subjected to SDS-PAGE using a 12% running gel, transferred to nitrocellulose membrane, incubated with an anti-ICAM-1 antibody for 24 h, and then incubated with an anti-rabbit horseradish peroxidase antibody for 1 h. The immunoreactive bands were detected by enhanced chemiluminescence reagents.

RT-PCR analysis. Total RNA was isolated using Trizol according to the protocol of the manufacturer. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification, as previously described (11). The primers used were as follows: ICAM-1: 5'-CAAAGGGGAGGTACCCCGAGGTG-3' (sense), 5'-TGCATGTCG-CCATTATGACTG-3' (anti-sense); β-actin: 5'-CTAGAAGCATTT-GCGTGGACGTAGGGA-3' (sense), 5'-TACGGGGTGTC-CCACACTGCGCCATC-3' (anti-sense).

Real-time PCR. Total RNA was extracted using Trizol reagent. mRNA was reverse transcribed into cDNA and analyzed by real-time RT-PCR. Real-time PCR was performed using SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ) and primers specific for ICAM-1 and GAPDH mRNAs. The levels of ICAM-1 expression were determined by normalizing to GAPDH expression.

Adhesion assay. HPAEpiCs (2 × 10⁶ cells/ml) were grown to confluence in six-well plates and incubated with 10 μg/ml LPS, and then adhesion assays were performed. Briefly, THP-1 cells (human acute monocytic leukemia cell line) were labeled with a fluorescent dye, 10 μM BCECF/AM, at 37°C for 1 h in RPMI-1640 medium (Gibco, Grand Island, NY) and subsequently washed by centrifugation. Confluent HPAEpiCs in six-well plates were incubated with THP-1 cells (2 × 10⁵ cells/ml) at 37°C for 1 h. Nonadherent THP-1 cells were removed, and plates were gently washed twice with PBS. The numbers of adherent THP-1 cells were determined by counting four fields per 200 high-power field well using a fluorescence microscope (Axiovert 200M; Zeiss, Jena, Germany). Experiments were performed in triplicate and repeated at least three times.

Isolation of cell fractions. HPAEpiCs (2 × 10⁶ cells/ml) were grown to confluence in 10-cm culture dishes and then incubated with 10 μg/ml LPS. Cells were harvested and then washed twice with ice-cold PBS, 300 μl of homogenization buffer A (20 mM Tris-HCl pH 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin, and 10 μg/ml leupeptin) was added to each dish, and the cells were scraped into a 1.5-ml tube with a rubber policeman. The suspension was sonicated for 5 s at output 1.5 with a sonicator (Misonix, Farmingdale, NY) and centrifuged at 5,000 g for 15 min at 4°C to pellet nuclei and other fragments. The supernatant can be retained as the cytoplasmatic fraction and was further centrifuged at 15,000 g for 60 min at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

Coimmunoprecipitation assay. HPAEpiCs (2 × 10⁶ cells/ml) were grown to confluence in 10-cm culture dishes and then incubated with 10 μg/ml LPS. Cells were harvested and then washed twice with ice-cold PBS, 300 μl of IP lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM NaF, 150 mM NaCl, and 1 mM Na3VO4) was added to each dish, and the cells were scraped into a 1.5-ml tube with a rubber policeman. The lysates containing 1 mg of protein were incubated with 2 μg of an anti-TRAF6 or anti-c-Src antibody at 4°C for 24 h, and then μl of 50% protein A-agarose beads was added and mixed at 4°C for 24 h. The immunoprecipitates were collected and washed thrice with a lysis buffer without Triton X-100. Laemmli buffer (5%) was added and subjected to electrophoresis on SDS-PAGE and then blotted using an anti-TR4, anti-MyD88, anti-c-Src, anti-TRAF6, anti-Rac1, or anti-p47(phox) antibody.

Transfection with siRNAs. Human siRNAs of scrambled, c-Src (SASI_HS01_00112905), TRAF6 (SASI_HS01_00116390), p42 (SASI_HS01_00124656), p65 (SASI_HS01_00171091), EGFR (SASI_HS01_00215449), PDGFRα (SASI_HS02_00341109), Akt (SASI_HS01_00205545), Nox2 (SASI_HS01_00086110), Nox4 (SASI_HS02_00349918), and p47(phox) (SASI_HS02_00302212) were from Sigma. Transient transfection of siRNAs (100 nM) was performed using a Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions.

Luciferase activity assay. The human ICAM-1 (pIC-339) firefly luciferase was kindly provided by Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). Additionally, the introduction of a mismatched primer mutation into the NF-κB to generate pGL3-ICAM-1 ΔNF-κB was performed, using the following (forward) primer: ΔNF-κB: 5'-GGTAGGCTTACTGGGCCCCC-AC-3'. All plasmids were prepared by using QIAGEN plasmid DNA preparation kits. ICAM-1-luc activity was determined as previously described using a luciferase assay system (Promega, Madison, WI).

Measurement of intracellular ROS accumulation. The intracellular H2O2 levels were determined by measuring fluorescence of DCFH-DA, and the O2− levels were determined by measuring the level of DHE. The fluorescence for dichlorofluorescin (DCF) and DHE staining was detected at 495/529 and 518/605 nm, respectively, using a fluorescence microscope (Zeiss, Axiovert 200M). For the purpose of these experiments, HPAEpiCs were washed with warm HBSS and incubated in HBSS or cell medium containing 10 μM DCFH-DA or DHE at 37°C for 45 min. Subsequently, HBSS or medium containing

Fig. 3. LPS induces ICAM-1 expression via NADPH oxidase/reactive oxygen species (ROS) in HPAEpiCs. A: cells were pretreated with Edaravone (Eda), diphenyleneiodonium dichloride (DPI), or apocynin (APO) for 1 h and then incubated with LPS for 16 h. The levels of ICAM-1 protein were determined by Western blot. B: cells were pretreated with Edaravone (10 μM), DPI (1 μM), or APO (100 μM) for 1 h and then incubated with LPS for 4 h (mRNA levels), 6 h (promoter activity), or 16 h (monocyte adhesion). The mRNA levels and promoter activity of ICAM-1 and the THP-1 cell adherence were measured. C and D: cells were pretreated with DPI (10 μM), Edaravone (10 μM), DPI (1 μM), or APO (100 μM) for 1 h and then treated with LPS for 30 min. C: ROS generation was measured. D: NADPH oxidase activity was measured. E: cells were pretreated with Edaravone (10 μM), DPI (1 μM), or APO (100 μM) for 1 h and then incubated with LPS for 30 min. Dihydroethidium fluorescence images were observed by a fluorescence microscope. Images shown are representatives of 3 independent experiments with similar results. F: cells were treated with LPS for the indicated time intervals. The cell lysates were subjected to immunoprecipitation using an anti-c-Src or anti-TRAF6 antibody. G: cells were transfected with scrambled, c-Src, or TRAF6 siRNA and incubated with LPS for 5 min. The cell lysates were subjected to immunoprecipitation using an anti-p47(phox) antibody. F and G: immunoprecipitates were analyzed by Western blot using an indicated antibody. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05; #P < 0.01 compared with the cells exposed to LPS alone.

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DCFH-DA or DHE was removed and replaced with fresh medium. HPAlEpiCs were then incubated with various concentrations of LPS. Cells were washed twice with PBS and detached with trypsin/EDTA, and the fluorescence intensity of the cells was analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA) at 518-nm excitation and 605-nm emission for DHE and at 495-nm excitation and 529-nm emission for DCF.

Determination of NADPH oxidase activity by chemiluminescence assay. HPAlEpiCs (2 × 10⁶ cells/ml) were grown to confluence in six-well plates and then incubated with 10 μg/ml LPS. After incubation, cells were gently scraped and centrifuged at 400 g for 10 min at 4°C. The cell pellet was resuspended with 35 μl/per well of ice-cold RPMI-1640 medium, and the cell suspension was kept on ice. To a final 200-μl volume of prewarmed (37°C) RPMI-1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (0.2 × 10⁶ cells) was added to initiate the reaction followed by immediate measurement of chemiluminescence in an Appliskan luminometer (Thermo Fisher Scientific, Rockford, IL) in out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 counts/min). Chemiluminescence was continuously measured for 12 min, and the activity of NADPH oxidase was expressed as counts per million cells.

Chromatin immunoprecipitation assay. To detect the in vivo association of transcription factors with human ICAM-1 promoter, chromatin immunoprecipitation (ChIP) analysis was performed as previously described (30). Briefly, HPAlEpiCs were cross linked with 1% formaldehyde at 37°C for 10 min and washed thrice with ice-cold PBS containing 1 mM PMSF and 1% aprotinin. Soluble chromatin was prepared using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s instructions and immunoprecipitated without (control) or with anti-p65 antibody and normal goat immunoglobulin G (IgG). Following washes and elution, immunopre-
cipitates were heated overnight at 65°C to reverse cross linking of DNA and protein. DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation. PCR fragments were analyzed on 2% agarose in 1× Tris-acetate-EDTA gel containing ethidium bromide.

Data analysis. Data were estimated using a GraphPad Prism Program (San Diego, CA). Quantitative data were expressed as the means ± SE and analyzed by one-way ANOVA followed by Tukey’s post hoc test. *P < 0.05 was considered significant. Data are expressed as means ± SE of three independent experiments.

RESULTS

LPS induces ICAM-1 expression via c-Src. To investigate the effect of LPS on ICAM-1 expression, cells were incubated with various concentrations of LPS for the indicated time intervals. As shown in Fig. 1A, LPS time and concentration dependently induced ICAM-1 protein expression in HPAEpiCs. On the other hand, the levels of ICAM-1 mRNA induced by 10 μg/ml LPS were determined by RT-PCR and real-time PCR. As shown in Fig. 1, B and C (open bar), LPS enhanced ICAM-1 mRNA expression in these cells. In addition, 10 μg/ml LPS also induced ICAM-1 promoter activity (Fig. 1C, shaded bar). Finally, we demonstrated that adhesion of THP-1 to HPAEpiCs challenged with LPS was enhanced, which was inhibited by an ICAM-1-neutralizing antibody (Fig. 1D).

c-Src has been shown to regulate ICAM-1 induction in various cell types (9, 16). In this study, we investigated whether c-Src activation was involved in LPS-induced ICAM-1 expression in HPAEpiCs. We found that pretreatment with the inhibitor of c-Src (PP1) significantly reduced LPS-induced ICAM-1 protein expression in these cells (Fig. 2A). To confirm the role of c-Src in LPS-induced ICAM-1 expression, as shown in Fig. 2B, transfection with c-Src siRNA attenuated LPS-induced ICAM-1 expression. In addition, pretreatment with PP1 also inhibited LPS-induced ICAM-1 mRNA expression and promoter activity (Fig. 2C). We further observed that enhanced adhesion of THP-1 to HPAEpiCs challenged with LPS was inhibited by pretreatment with PP1 (Fig. 2D).

On the other hand, LPS could stimulate c-Src phosphorylation, which was inhibited by pretreatment with PP1 or transfection with c-Src siRNA during the period of observation (Fig. 2E), suggesting that c-Src activation plays a key role in LPS-induced inflammatory responses.

TLRs recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades, in which MyD88 and TRAF6 are key adaptors (11). Previous study also showed that lipoteichoic acid (LTA) induced the formation of a TLR2/MyD88/TRAF6/c-Src complex in human airway smooth muscle cells (11). Thus we further investigated the physical association of TLR4, MyD88, TRAF6, and c-Src in LPS-induced ICAM-1 expression by immunoprecipitation using anti-c-Src and anti-TRAF6 antibodies subjected to Western blot. As shown in Fig. 2F, LPS induced the formation of a TLR4/MyD88/TRAF6/c-Src complex in these cells. The association of these components was further confirmed by transfection with c-Src or TRAF6 siRNA (Fig. 2G). These results suggested that LPS-induced ICAM-1 expression is mediated through the formation of a TLR4/MyD88/TRAF6/c-Src complex in HPAEpiCs.

Involvement of NADPH oxidase and ROS generation in LPS-induced ICAM-1 expression. Several lines of evidence have demonstrated that ROS contribute to ICAM-1 expression induced by different stimuli in various cell types (8, 34). In addition, NADPH oxidase is an important enzymatic source for the production of ROS under various pathologic conditions. Thus the role of NADPH oxidase in ROS generation associated with ICAM-1 expression in response to LPS was investigated. As shown in Fig. 3A, pretreatment with NADPH oxidase inhibitors (DPI and APO) or a ROS scavenger (Edaravone) concentration dependently attenuated LPS-induced ICAM-1 protein expression. Moreover, pretreatment with Edaravone, DPI, or APO also inhibited ICAM-1 mRNA expression and promoter activity as well as monocyte adhesion induced by LPS (Fig. 3B). Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of p47^{phox}, p67^{phox}, and p40^{phox}. Phosphorylation of p47^{phox} leads to a conformational change allowing its interaction with p22^{phox} (11). It has been demonstrated that p47^{phox} organizes the translocation of other cytosolic factors, hence its designation as “organizer subunit” (11). To determine whether NADPH oxidase and p47^{phox} are involved in ICAM-1 expression, as shown in (Fig. 4A), transfection with siRNA of p47^{phox}, Nox2, or Nox4 markedly attenuated LPS-induced ICAM-1 protein levels. Indeed, LPS time dependently induced NADPH oxidase activation and intracellular ROS generation in these cells (Fig. 4B), which were reduced by Edaravone, DPI, or APO (Fig. 3, C and D). c-Src has been shown to regulate NADPH oxidase activation and ROS generation in response to LTA (11). Thus we also observed that pretreatment with PP1 inhibited LPS-induced NADPH oxidase activation and intracellular ROS generation (Fig. 3, C and D). In addition, we used DHE to measure the levels of superoxide production induced by LPS. As shown in Fig. 3E, LPS-induced superoxide generation was reduced by Edaravone, DPI, or APO in these cells.

Previous study showed the requirement of p47^{phox} for Rac1-dependent NADPH oxidase in the cardiovascular and dippogenic actions of angiotensin II in the brain (35). First, we found that LPS stimulated the recruitment of p47^{phox} from the cytosolic to the membrane fraction (Fig. 4C). Thus we further investigated the physical association of TRAF6, c-Src, Rac1, and p47^{phox} in LPS-induced NADPH oxidase activation. As shown in Fig. 3F, cells were incubated with LPS for the indicated time intervals. The cell lysates were subjected to immunoprecipitation using an anti-c-Src or anti-TRAF6 antibody and then analyzed by Western blot using an antibody as indicated. The protein levels of p47^{phox} and Rac1 were increased in a c-Src- or TRAF6-immunoprecipitated complex (Fig. 3F), which were attenuated by transfection with c-Src or TRAF6 siRNA (Fig. 3G). In addition, transfection with Nox2 or Nox4 siRNA had no effect on LPS-stimulated c-Src phosphorylation (Fig. 4D), suggesting that c-Src is an upstream component of NADPH oxidase/ROS. These results demonstrated that LPS induced ICAM-1 expression and NADPH oxidase activation via the formation of a TRAF6/c-Src/p47^{phox}/Rac1 complex in HPAEpiCs.

LPS stimulates PDGFR- and EGFR-dependent ICAM-1 induction. EGFR and PDGFR have been shown to regulate ICAM-1 induction in various cell types (16, 33). In addition, c-Src plays a key role in mediating PDGFR and EGFR trans-activation (6, 31). Here, we found that LPS induced ICAM-1
protein, mRNA expression, and promoter activity, which were attenuated by pretreatment with the inhibitor of PDGFR (AG1296) or EGFR (AG1478) (Fig. 5, A and B). Enhanced adhesion of THP-1 to HPAEpiCs challenged with LPS was also inhibited by AG1296 or AG1478 (Fig. 5C). The roles of EGFR and PDGFR in these responses were further confirmed by transfection with EGFR or PDGFR siRNA, which markedly inhibited LPS-induced ICAM-1 expression (Fig. 5D). We
in HPAEpiCs. In this study, we investigated the role of cell survival, differentiation, metabolism, proliferation, and p42/p44 MAPK has been shown to participate in the regulation of p42/p44 MAPK phosphorylation in LPS-induced ICAM-1 expression. As shown in Fig. 7A, pretreatment with the inhibitor of MEK1/2 (U0126) markedly attenuated LPS-induced ICAM-1 protein expression. To confirm the role of p42/p44 MAPK in LPS-induced ICAM-1 expression, as shown in Fig. 7B, transfection with p42 siRNA markedly attenuated LPS-induced ICAM-1 expression. In addition, pretreatment with U0126 also inhibited LPS-induced ICAM-1 mRNA expression and promoter activity (Fig. 7C). We further found that enhanced adhesion of THP-1 to HPAEpiCs challenged with LPS was also inhibited by U0126 (Fig. 7D). On the other hand, LPS could stimulate p42/p44 MAPK phosphorylation, which was inhibited by pretreatment with U0126 or transfection with p42 siRNA during the period of observation (Fig. 7E). Moreover, we also found that LPS-stimulated p42/p44 MAPK phosphorylation was inhibited by PP1, AG1296, AG1478, DPI, APO, LY294002, or Edaravone (Fig. 7E). In addition, transfection with Nox2, Nox4, PDGFR, EGFR, or Akt siRNA also suppressed LPS-stimulated p42/p44 MAPK phosphorylation (Fig. 7F). These results suggested that LPS-induced ICAM-1 expression is mediated through a c-Src/NADPH oxidase/ROS/PDGFR and EGFR pathway in HPAEpiCs.

LPS induces ICAM-1 expression via PI3K/Akt. PI3K and Akt have been shown to mediate cellular activation, proliferation, and migration in various cell types, suggesting that they play critical roles in the development of inflammation (12). In this study, we investigated the roles of PI3K/Akt in LPS-induced ICAM-1 expression. As shown in Fig. 6, A and B, pretreatment with the inhibitor of PI3K (LY294002) markedly attenuated LPS-induced ICAM-1 protein, mRNA expression, and promoter activity. Enhanced adhesion of THP-1 to HPAEpiCs challenged with LPS was also inhibited by LY294002 (Fig. 6C). The role of Akt in this response was further confirmed by transfection with Akt siRNA, which markedly inhibited LPS-induced ICAM-1 expression (Fig. 6D). On the other hand, we also found that LPS-stimulated Akt phosphorylation was reduced by pretreatment with AG1296, AG1478, LY294002, DPI, APO, or Edaravone during the period of observation (Fig. 6E). In addition, transfection with Nox2, Nox4, PDGFR, EGFR, or Akt siRNA also attenuated the LPS-stimulated Akt phosphorylation, but Akt siRNA had no effect on c-Src, EGFR, and PDGFR phosphorylation (Figs. 4D, 5G, and 6F). These results suggested that LPS-induced ICAM-1 expression is mediated through Akt activation via a c-Src/NADPH oxidase/ROS/PDGFR and EGFR pathway in HPAEpiCs.

LPS induces ICAM-1 expression through p42/p44 MAPK. p42/p44 MAPK has been shown to participate in the regulation of various processes, including cell adhesion, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription (26). In this study, we investigated the role of p42/p44 MAPK in LPS-induced ICAM-1 expression. As shown in Fig. 7A, pretreatment with the inhibitor of MEK1/2 (U0126) markedly attenuated LPS-induced ICAM-1 protein expression. To confirm the role of p42/p44 MAPK in LPS-induced ICAM-1 expression, as shown in Fig. 7B, transfection with p42 siRNA markedly attenuated LPS-induced ICAM-1 expression. In addition, pretreatment with U0126 also inhibited LPS-induced ICAM-1 mRNA expression and promoter activity (Fig. 7C). We further found that enhanced adhesion of THP-1 to HPAEpiCs challenged with LPS was also inhibited by U0126 (Fig. 7D). On the other hand, LPS could stimulate p42/p44 MAPK phosphorylation, which was inhibited by pretreatment with U0126 or transfection with p42 siRNA during the period of observation (Fig. 7E). Moreover, we also found that LPS-stimulated p42/p44 MAPK phosphorylation was inhibited by PP1, AG1296, AG1478, DPI, APO, LY294002, or Edaravone (Fig. 7E). In addition, transfection with Nox2, Nox4, PDGFR, EGFR, or Akt siRNA also suppressed LPS-stimulated p42/p44 MAPK phosphorylation (Fig. 7F). These results suggested that LPS-induced ICAM-1 expression is mediated through a c-Src/NADPH oxidase/ROS/PDGFR and EGFR pathway in HPAEpiCs.
Fig. 6. LPS induces ICAM-1 expression via phosphatidylinositol-3-kinase (PI3K)/Akt in HPAEpiCs. A: cells were pretreated with LY294002 for 1 h and then incubated with LPS for 16 h. The levels of ICAM-1 protein were determined by Western blot. B: cells were pretreated with LY294002 (3 μM) for 1 h and then incubated with LPS for 4 h (mRNA levels) or 6 h (promoter activity). The mRNA levels of ICAM-1 were determined by real-time PCR (open bars). The promoter activity of ICAM-1 was determined by promoter assay (shaded bars). C: cells were pretreated with LY294002 for 1 h, incubated with LPS for 16 h, and then measured for THP-1 cell adherence. D: cells were transfected with scrambled or Akt siRNA and incubated with LPS for 16 h. The levels of ICAM-1 protein were determined by Western blot. E: cells were pretreated without or with AG1296, AG1478, LY294002, DPI, APO, or Edaravone for 1 h and then incubated with LPS for the indicated time intervals. F: cells were transfected with scrambled or Akt siRNA and incubated with LPS for the indicated time intervals. E and F: levels of phospho-Akt, phospho-Src, phospho-PDGFR, and phospho-EGFR were determined by Western blot. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05, #P < 0.01 compared with the cells exposed to LPS alone.
Fig. 7. LPS induces ICAM-1 expression via p42/p44 MAPK in HPAEpiCs. A: cells were pretreated with U0126 for 1 h and then incubated with LPS for 16 h. The levels of ICAM-1 protein were determined by Western blot. B: cells were transfected with either scrambled or p42 siRNA and then incubated with LPS for 16 h. The levels of p42 and ICAM-1 protein were determined by Western blot. C: cells were pretreated with U0126 for 1 h and then incubated with LPS for 4 h (mRNA levels) or 6 h (promoter activity). The mRNA levels of ICAM-1 were determined by real-time PCR (open bars). The promoter activity of ICAM-1 was determined by promoter assay (shaded bars). D: cells were pretreated with U0126 for 1 h, incubated with LPS for 16 h, and then measured for THP-1 cell adherence. E: cells were pretreated with U0126, PP1, AG1296, AG1478, LY294002, Edaravone, or DPI for 1 h, transfected with p42 siRNA, and then incubated with LPS for the indicated time intervals. E and F: levels of phospho-p42/p44 MAPK were determined by Western blot. Data are expressed as means ± SE of 3 independent experiments. #P < 0.01 compared with the cells exposed to LPS alone.
ment with PP1, AG1296, AG1478, Edaravone, DPI, LY294002, or U0126 (Fig. 8G). The roles of these signaling components in LPS-mediated responses were further confirmed by transfection with siRNA of c-Src, PDGFR, EGFR, Akt, p47phox, Nox2, Nox4, p42, or p65, which significantly reduced LPS-induced ICAM-1 mRNA levels and NF-κB promoter activity (Fig. 9). Taken together, these data suggest that LPS-induced ICAM-1 expression is mediated through NF-κB via a c-Src/NADPH oxidase/ROS/EGFR and PDGFR/PI3K/Akt/p42/p44 MAPK pathway in HPAEpiCs.
DISCUSSION

Asthma and chronic obstructive pulmonary disease (COPD) are pulmonary disorders characterized by various degrees of inflammation and tissue remodeling. LPS has been shown to induce proinflammatory cytokines in the lung via the TLR4/CD14 signaling cascade (1). In addition, a central feature of the pathophysiology of acute inflammation and septic shock triggered by LPS is the production of multiple proinflammatory mediators, such as cellular adhesion molecules (CAMs). Several clinical investigations have demonstrated that the expression of ICAM-1 on the bronchial epithelium was increased in patients with COPD (4). However, the molecular mechanisms by which LPS induces ICAM-1 expression are not fully understood in HPAEpiCs. The present study clearly demonstrated that ICAM-1 expression induced by LPS was mediated through a TLR4/MyD88/c-Src/NADPH oxidase/ROS/EGFR and PDGFR/Pi3K/Akt/p42/p44 MAPK/NF-κB pathway. Genetic silencing through transfection with siRNA of c-Src, Nox2, Nox4, PDGFR, EGFR, Akt, p42, or p65 or pretreatment with the inhibitor of c-Src, NADPH oxidase, ROS, EGFR, PDGFR, PI3K, MEK1/2, or NF-κB abrogated LPS-induced ICAM-1 expression and monocyte adhesion. The results obtained with p65 siRNA indicate that c-Src and other phosphorylated components are upstream of NF-κB activated by LPS. Moreover, LPS induced the formation of a TLR4/MyD88/TrAF6/c-Src/p47phox/Rac1 complex, which further promoted NADPH oxidase activation and ROS generation.

The LPS of Gram-negative bacterium is a well-known inducer of the innate immune responses. TLR4 and myeloid differentiation factor 2 form a heterodimer that recognizes a common “pattern” in structurally diverse LPS molecules (27). In addition, TLR4 is necessary for signal transduction induced by LPS and such cellular consequences of LPS stimulation as expression of ROS and various cytokines (24). Adhesion molecules likely play important roles in maintaining the normal cellular interactions.
Fig. 10. LPS induces NF-κB p65 phosphorylation and translocation via a c-Src-dependent pathway. A: cells were pretreated with Edaravone, DPI, APO, U0126, LY294002, or PP1 for 1 h and then treated with LPS for 30 min. The CE and NE fractions were prepared and analyzed by Western blot using an anti-p65 antibody. GAPDH and lamin A were used as marker proteins for cytosolic and nuclear fractions, respectively. B: cells were preincubated without or with Edaravone, DPI, APO, PP1, AG1296, AG1478, LY294002, U0126, or Bay11-7082 for 1 h and then treated with LPS for the indicated time intervals. C: cells were transfected with scrambled, c-Src, PDGFR, EGFR, Akt, p47phox, Nox2, Nox4, p42, or p65 siRNA and incubated with LPS for 15 min. B and C: levels of phospho-p65 were determined by Western blot. D: cells were treated with LPS for the indicated time intervals or pretreated with AG1296, AG1478, LY294002, or U0126 for 1 h and then incubated with LPS for 30 min. The NF-κB p65 binding activities were analyzed by a chromatin immunoprecipitation assay. Data are representatives of 3 independent experiments with similar results.
LPS induces ROS generation and ICAM-1 expression

mal structure and function of the lung, as well as participating in pulmonary processes, such as inflammation (12). In our study, we established that LPS potentially enhanced ICAM-1 expression and THP-1 adhesion to HPAEpiCs. Moreover, we suggested that these responses induced by LPS may promote pulmonary diseases.

c-Src is a member of the highly conserved Src family of nonreceptor tyrosine kinases, which display different expression patterns and have been implicated in numerous cellular processes, such as innate immune responses and signalings induced by cytokines, antigens, and growth factors (12, 13). LPS has been shown to initiate c-Src activation in various cell types (12, 13, 18). This notion is confirmed by our observation that inhibition of c-Src by PP1 or c-Src siRNA attenuated THP-1 adhesion to HPAEpiCs challenged with LPS via down-regulation of ICAM-1 expression. We also suggested that LPS-stimulated c-Src phosphorylation was involved in LPS-enhanced inflammation, which was inhibited by Src inhibitor but not by APO, DPI, Edaravone, AG1296, AG1478, LY294002, U0126, or Bay11-7082, suggesting that c-Src is an upstream component of these signaling molecules in LPS-mediated responses (data not shown). TLRs recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades, in which MyD88 and TRAF6 are key adaptor proteins (11). A previous study also showed that LTA induced the formation of a TLR2/MyD88/TRAF6/c-Src complex in human airway smooth muscle cells (11). However, in this study, we found that LPS enhanced the formation of a TLR4/MyD88/TRAF6/c-Src complex, which was attenuated by transfection with c-Src or TRAF6 siRNA in HPAEpiCs. On the other hand, c-Src has been shown to regulate NADPH oxidase activation and ROS generation in response to LTA (11). Several lines of evidence have demonstrated that ROS contribute to ICAM-1 expression in various cell types (13). In addition, NADPH oxidase is an important enzymatic source for the production of ROS under various pathological conditions. Indeed, in HPAEpiCs, we found that inhibition of NADPH oxidase and ROS significantly reduced LPS-induced ICAM-1 expression and THP-1 cell adhesion. We further demonstrated that LPS-induced NADPH oxidase activation and intracellular ROS generation were mediated via a c-Src-dependent pathway. Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of p47phox, p67phox, and p40phox. p47phox is a major regulator of NADPH oxidase. In the resting state, it localizes in the cytoplasm, where its activity is inhibited through an autoinhibitory phosphorylation (9). However, upon stimulation, p47phox interacts with cytosolic subunits p67phox and p40phox and the complex translocates to the cell membrane to assemble the active oxidase, which generates superoxide anion. This process is triggered by phosphorylation of p47phox, which is the key factor for activation of NADPH oxidase and consequent ROS production. In this study, LPS-induced ICAM-1 expression was attenuated by transfection with Nox2, Nox4, or p47phox siRNA, suggesting the involvement of NADPH oxidase and ROS production in these responses. A previous study showed the requirement of p47phox for Rac1-dependent NADPH oxidase in the cardiovascular and dyspogenic actions of angiotensin II in the brain (35). Here, we observed that LPS induced ICAM-1 expression and NADPH oxidase activation via the formation of a TRAF6/c-Src/p47phox/Rac1 complex, which was attenuated by transfection with TRAF6 or c-Src siRNA. The detailed protein-protein interactions among TRAF6, c-Src, p47phox, and Rac1 were not known. Our results are the first to show a novel role of TRAF6/c-Src/p47phox/Rac1 complex formation in LPS-stimulated ICAM-1 expression in HPAEpiCs. In the future, we hope to further determine which domains of TRAF6, c-Src, p47phox, and Rac1 are involved in protein-protein interactions caused by LPS.

It has been reported that several G protein-coupled receptors initiate the PI3K/Akt pathway through transactivation of the EGFR or PDGFR in various cell types (3, 16, 31). However, little was known about the mechanisms of LPS that initiate the expression of ICAM-1 mediated through c-Src-dependent transactivation of EGFR or PDGFR in HPAEpiCs. Here, we established that LPS markedly induced ICAM-1 expression via a PDGFR- or EGFR-dependent pathway by using the inhibitor of PDGFR or EGFR and PDGFR or EGFR siRNA. In addition, in these cells, c-Src activation and NADPH oxidase/ROS production were involved in LPS-induced PDGFR and EGFR transactivation. Thus, LPS-induced ICAM-1 expression and monocyte adherence were mediated through the c-Src/NADPH oxidase/ROS-dependent PDGFR and EGFR activation. Recent studies have suggested that numerous components of the PI3K pathway play a crucial role in the expression and activation of inflammatory mediators, inflammatory cell recruitment, and immune cell function (12). It has been established that growth factors, such as EGF and PDGF, stimulate Akt activation, which is inhibited by Wortmannin, LY294002, or overexpression of the dominant-negative mutant of PI3K (2). Akt has also been implicated in the pathogenesis of
inflammatory responses (12). This notion is confirmed by our observation that inhibition of PI3K/Akt by using LY294002 attenuated THP-1 adhesion to HPAEpiCs challenged with LPS via downregulation of ICAM-1 expression. We also demonstrated that LPS-enhanced Akt phosphorylation was involved in pulmonary inflammation, which was inhibited by PP1, Edaravone, DPI, AG1296, AG1478, or LY294002 but not by U0126 and Bay11-7082, suggesting that Akt is an upstream component of p42/p44 MAPK and NF-κB and downstream component of c-Src/NADPH oxidase/ROS/EGFR and PDGFR in LPS-mediated responses (data not shown). On the other hand, we established that the c-Src/NADPH oxidase/ROS/EGFR and PDGFR pathway plays a key role in mediating LPS-induced Akt activation in HPAEpiCs. The MAPK family consists of three major members: p42/p44 MAPK, p38 MAPK, and JNK1/2. MAPKs are important intracellular signalings and play critical roles in regulating inflammatory responses (12). LPS has been shown to mediate p42/p44 MAPK, p38 MAPK, and JNK1/2 activation (10, 29). Moreover, p42/p44 MAPK has been shown to participate in the regulation of a large variety of processes, including cell adhesion, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription (26). Here, we found that inhibition of p42/p44 MAPK by using U0126 or p42 siRNA attenuated THP-1 adhesion to HPAEpiCs challenged with LPS via downregulation of ICAM-1 expression. In addition, we also observed that LPS-induced p42/p44 MAPK activation was mediated through a c-Src/NADPH oxidase/ROS/EGFR and PDGFR/PI3K/Akt pathway. Although inhibition of p38 MAPK or JNK1/2 decreased LPS-induced ICAM-1 expression in HPAEpiCs, inhibition of EGFR, PDGFR, and PI3K/Akt had no effect on p38 MAPK and JNK1/2 activation (data not shown). Thus, in this study, we focus on the critical role of p42/p44 MAPK in LPS-mediated ICAM-1 expression.

NF-κB, as a transcription factor, is linked to the expression of various genes and plays an essential role in immune responses.
and inflammatory responses (12). Abnormal NF-κB signaling results in human diseases, such as inflammation and various cancers. Therefore, regulation of NF-κB activity may be useful for the treatment or improve the symptoms of human inflammatory disorders (12, 13). Moreover, in our study, we also found that NF-κB plays an important role in mediating LPS-enhanced ICAM-1 expression and monocyte adherence by using the inhibitor of NF-κB and p65 siRNA in HPAEpiCs. On the other hand, we established that LPS stimulated p65 phosphorylation through a c-Src/NADPH oxidase pathway, which may be associated with pathogenesis of pulmonary diseases. Unlike other transcription factors, NF-κB proteins mainly reside in the cytoplasm in an inactive form bound to an inhibitory protein referred to as IκB. Upon appropriate stimulation, activated NF-κB is rapidly released from the cytoplasmic complex by phosphorylation and ubiquitination-dependent degradation of IκB. The IκB-released NF-κB dimer translocates into the nucleus, where it binds cognate DNA sequences and activates transcription of specific target genes (28). This notion is confirmed by our observation that LPS induced p65 translocation, IκBα degradation and turns on the ICAM-1 gene transcription.

Several studies indicate that PKCs are involved in intracellular TLR signal transduction and activate the innate immune system leading to cytokine secretion. Further studies have shown that inhibition of PKC-α and PKC-δ could retard the inflammatory responses in lung injury models (19). In this study, we found that inhibition of PKC with H7 decreased ICAM-1 expression induced by LPS (Fig. 12A). In addition, transfection with either PKC-α or PKC-δ siRNA attenuated the LPS-stimulated p42/p44 MAPK and p65 phosphorylation but had no effect on c-Src and Akt phosphorylation (Fig. 12, B and C), indicating that PKC-α or PKC-δ is a downstream component of c-Src and Akt and an upstream component of p42/p44 MAPK and NF-κB in LPS-mediated cascade. These results suggested that LPS-induced ICAM-1 expression is mediated through PKC-α/PKC-δ-dependent p42/p44 MAPK/NF-κB in these cells. Therefore, the role of PKCs in LPS-induced ICAM-1 expression is an important issue and needs to be investigated in the future.

In summary, as depicted in Fig. 13, these findings concerning LPS-induced ICAM-1 expression and monocyte adhesion
imply that LPS might play an important role in lung inflammatory diseases, mediated through a TLR4/MyD88/TRAF6/c-Src/NADPH oxidase/ROS/PDGFR- and EGFR/PI3K/Akt/p42/p44 MAPK/NF-κB-dependent signaling pathway in HPAEp-ICs. Furthermore, the knockdown/mutation of downstream signal molecules failed to block and/or reduce the phosphorylation of upstream signal molecules (data not shown). These results indicate a role for HPAEpICs, in addition to their physiological function, as inflammatory cells involved in the production of inflammatory mediators, which may contribute to the inflammatory responses in various lung diseases.

REFERENCES

