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Lipoteichoic Acid Induces HO-1 Expression via the TLR2/MyD88/c-Src/NADPH Oxidase Pathway and Nrf2 in Human Tracheal Smooth Muscle Cells¹

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Heme oxygenase (HO)-1 is a stress-inducible rate-limiting enzyme in heme degradation that confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis. Increasing reports have indicated that lipoteichoic acid (LTA) exerts as LPS as an immune system-stimulating agent and plays a role in the pathogenesis of severe inflammatory responses induced by Gram-positive bacterial infection. We report that LTA is an inducer of HO-1 expression mediated through the signaling pathways in human tracheal smooth muscle cells (HTSMCs). LTA-induced HO-1 protein levels, mRNA expression, and promoter activity were attenuated by transfection with dominant negative mutants of TLR2 and MyD88, by pretreatment with the inhibitors of c-Src (PP1), NADPH oxidase (diphenylene iodonium chloride (DPI) and apocynin (APO)), and reactive oxygen species (ROS) scavenger (N-acetyl-l-cysteine) or by transfection with small interfering RNAs of Src and NF-E2-related factor 2 (Nrf2). LTA-stimulated translocation of p47^{phox} and Nrf2 and ROS production was attenuated by transfection with dominant negative mutants of TLR2, MyD88, and c-Src and by pretreatment with DPI or APO. Furthermore, LTA-induced TLR2, MyD88, TNFR-associated factor (TRAF)6, c-Src, and p47^{phox} complex formation was revealed by immunoprecipitation using an anti-TLR2 or anti-c-Src Ab followed by Western blot analysis against an anti-TLR2, anti-MyD88, anti-TRAF6, anti-c-Src, or anti-p47^{phox} Ab. These results demonstrated that LTA-induced ROS generation was mediated through the TLR2/MyD88/TRAF6/c-Src/NADPH oxidase pathway, in turn initiates the activation of Nrf2, and ultimately induces HO-1 expression in HTSMCs. The Journal of Immunology, 2008, 181: 5098–5110.

Airway smooth muscle is considered as an end-response effector regulating regional differences in ventilation by contracting in response to various neurotransmitters, proinflammatory mediators, and exogenous substances released under homeostatic or pathologic conditions, such as asthma (1). When airway cells and tissues are exposed to oxidative stress elicited by inflammatory reactions, elevated levels of reactive oxygen species (ROS) can trigger a variety of deleterious effects within the airways. NADPH oxidase is an enzymatic source for the production of ROS under various pathologic conditions.

Lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria, is an amphiphilic, negatively charged glycolipid (2). Recently, increasing reports have indicated that LTA shares many inflammatory properties of LPS and plays a role in the pathogenesis of septic shock or severe inflammatory responses induced by Gram-positive bacterial infection (3). Airway inflammation develops in the context of innate immune cells that express TLRs. TLRs recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades, in which MyD88 and TNFR-associated factor (TRAF)6 are key adaptor proteins (4). A recent study using highly purified preparations of *Staphylococcus aureus* LTA has clearly shown that staphylococcal LTA can efficiently stimulate monocytes via TLR2 to produce TNF-α (5). Another study has also shown that LPS-dependent activation of NADPH oxidase is mediated by a direct interaction of TLR4 with this enzyme (6). However, whether LTA-induced NADPH oxidase activation in human tracheal smooth muscle cells (HTSMCs) is still unknown.

Heme oxygenase (HO)-1 is the key enzyme responsible for the degradation of heme to carbon monoxide, free iron, and biliverdin-IXα (7). In mammals, biliverdin-IXα is further converted to bilirubin-IXα, an endogenous radical scavenger (8), with recently recognized anti-inflammatory properties (9). However, the release of free iron is rapidly sequestered into the iron storage protein ferritin, leading to additional antioxidant (10) and antiapoptotic (11) effects. Carbon monoxide exerts several biological functions including antiapoptotic and anti-inflammatory properties (12). HO-1 is induced by various stimuli, such as LPS, proinflammatory cytokines, and oxidants (13–15). A recent study has shown that TLR2 contributes to HO-1 expression in traumatic brain injury (16). In addition, HO-1 expression is up-regulated in the airways of patients with asthma (17). Airway smooth muscle contractility, which can be increased through ROS formation, is inhibited by HO-1 induction (9). Thus, the increase in HO-1 expression by

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3 Abbreviations used in this paper: ROS, reactive oxygen species; HO-1, heme oxygenase-1; LTA, lipoteichoic acid; sRNA, small interfering RNA; DPI, diphenylene iodonium chloride; APO, apocynin; ARE, antioxidant response element; TRAF, TNFR-associated factor; HTSMC, human tracheal smooth muscle cell; β-gal, β-galactosidase; Nrf, NF-E2-related factor; NAc, N-acetyl-l-cysteine.

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these stress stimuli is thought to be an adaptive mechanism that protects the cells from oxidative damage. However, whether LTA induced HO-1 expression via TLR2 in HTSMCs is also still unknown.

Given the potential physiological importance of HO-1 in mediating cellular homeostasis as a general inducible stress protein response, a considerable research effort has focused on characterizing the molecular mechanisms that regulate the transcriptional activity of the HO-1 gene. c-Src, a common modular participating in the cross-talk between the cytoplasmic protein tyrosine kinases and receptors, has been shown to mediate LTA signaling in murine macrophages (3). In addition, the Src family kinases have been shown to regulate NADPH oxidase activation and ROS production (18). Moreover, the 5’ untranslated region of the human HO-1 gene contains many stress-activated response elements, such as antioxidant response elements (AREs). NF-E2-related factor 2 (Nrf2) is an important transcription factor that regulates expression of antioxidant defense genes through binding to AREs in the promoter region, such as HO-1 in response to ROS (19). Nrf2 resides in the cytosol bound to an inhibitor Keap 1. Upon activation, Nrf2 is phosphorylated, released from Keap 1, and then translocated into the nucleus (20). In this study, we investigated the roles of c-Src and Nrf2 in regulating LTA-induced HO-1 expression.

Therefore, LTA may play a potential role in regulation of specific gene expression, such as HO-1 and thereby prevent inflammatory responses. However, the mechanisms of intracellular signaling pathways involved in LTA-induced HO-1 expression in HTSMCs are unclear. In this study, the mechanisms underlying LTA-induced HO-1 expression in HTSMCs were studied. Our results demonstrated that LTA induced HO-1 expression in a TLR2/MyD88/TRAF6/c-Src/NADPH oxidase and Nrf2-dependent manner in HTSMCs.

Materials and Methods

Materials

PP1 and diphenylethylenenitrone chloride (DPI) were from Biomol. QuikChange Site-Directed Mutagenesis kit and GeneJammer transfection reagent were from Stratagene. Metfectene transfection reagent was from Biontex. Luciferase assay kit was from Promega. Anti-TLR2 and anti-Nrf2, anti-MyD88, anti-TRAF6, and anti-p47(phox) Abs were from Santa Cruz Biotechnology. Phosho-Src (Tyr416) Ab was from Cell Signaling Technology. Anti-GAPDH antibody was from Cell Signaling Technology. The DCP-DA (2’7’-dichlorofluorescein diacetate) was from Molecular Probes. LTA from S. aureus (L2515) and N-acetyl-l-cysteine (Nac) were from Sigma-Aldrich. APO was purchased from ChromaDex.

Cell culture

The HTSMCs were purchased from ScienCell Research Laboratory and cultured as previously described (21). Experiments were performed with cells from passages 3 to 8. Before treatment or stimulation with reagents, cells were serum starved for 24 h.

Preparation of cell extracts and Western blot analysis

Growth-arrested HTSMCs were incubated with LTA at 37°C for the indicated times. 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 (21). Samples were denatured, subjected to SDS-PAGE using a 12% running gel, and transferred to nitrocellulose membrane. Membranes were incubated with anti-HO-1 Ab for 24 h, and then membranes were incubated with anti-goat HRP Ab for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

Total RNA extraction and RT-PCR analysis

Total RNA was isolated from HTSMCs in 10-cm culture dishes with TRIzol according to the protocol of the manufacturer. RNA concentration was spectrophotometrically determined at 260 nm. First strand cDNA synthesis was performed with 2 μg of total RNA using random hexamers as primers in a final volume of 20 μl (5 μg/μl random hexamers, 1 mM dNTPs, 2 U/μl RNAsin, and 10 μl/Moloney murine leukemia virus reverse transcriptase). The reaction was conducted at 37°C for 60 min. cDNAs encoding β-actin and HO-1 were amplified from 3 to 5 μl of the cDNA reaction mixture using specific gene primers. The primers used were as follows: β-actin 5’-TGAAGGGGTCAACCCACCTGGCCCATCTA-3’ (sense) and 5’-CATAGAGATCCTTGCGAGCATGATG-3’ (antisense) and HO-1 5’-CAGCCCCACGAGATTG-3’ (sense) and 5’-AGCTGAGTGTTAGCAGCCA-3’ (antisense). The amplification profile includes 1 cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C (for β-actin) or 58°C (for HO-1) for 1 min, and extension at 72°C for 1 min then 1 cycle of final extension at 72°C for 5 min. The expression of β-actin was used as an internal control.

Transient transfection with small interfering RNAs (siRNAs) and dominant negative plasmids

SMARTpool RNA duplexes corresponding to human Src, MyD88, TRAF6, Nrf2, and scrambled no. 2 siRNA were from Dharmacon. HTSMCs (passage 4 or 5) were cultured on 12-well plates. At 70–80% confluence, transient transfection of siRNAs was conducted using Metafectene transfection reagent. Briefly, siRNA (100 nM) was formulated with Metafectene transfection reagent according to the manufacturer’s instructions. The transfection complex was diluted into 400 μl of DMEM/F-12 medium and added directly to the cells. The medium was replaced with serum-free DMEM/F-12 after 2 h. Cells were analyzed at 72 h after transfection by Western blot analysis. The transfection efficiency (70–80%) was determined by transfection with enhanced GFP. The plasmids encoding dominant negative mutants of c-Src (K295M), TLR2, and MyD88 were provided by Dr. C. C. Chen (Department of Pharmacology, National Taiwan University, Taipei, Taiwan), Dr. J. Han (Department of Immunology, The Scripps Research Institute, La Jolla, CA), and Dr. R. Lee (Section of Immunology, Yale School of Medicine, New Haven, CT). All plasmids were prepared by using Qiagen plasmid DNA preparation kits. HTSMCs were plated in 6-well plates, reaching ~80% confluence. The Metafectene transfection reagent was prepared according to the instructions of manufacturer. The amount of plasmid transfected was kept constant (2 μg of dominant negative mutants of c-Src, TLR2, and MyD88 for each well). The Metafectene transfection reagent and dominant negative plasmids were mixed for 30 min. The complex of Metafectene transfection reagent and dominant negative plasmids were added to each well, and then incubated at 37°C for 24 h. After 24 h of transfection, the cells were washed once with PBS and maintained in DMEM/F-12 containing 1% FBS before treatment with LTA.

Measurement of HO-1 luciferase activity

For construction of the HO-1 luciferase (luc) plasmid, human HO-1 promoter, a region spanning −3106 to +186 bp provided by Dr. Y. C. Liang (Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan) was cloned into a pGL2-basic vector (Promega). HO-1-luc plasmid was transiently transfected into HTSMCs using GeneJammer transfection reagent. Briefly, HO-1-luc plasmid and β-galactosidase (β-gal) were formulated with GeneJammer transfection reagent. The transfection complex was diluted into 900 μl of DMEM/F-12 medium, and then added directly to the cells. After transfection for 6 h, the medium was replaced with DMEM/F-12 medium containing 10% FBS. After incubation for 24 h, cells were starved for 24 h in DMEM/F-12 medium with 1% FBS. After starvation, cells were treated with LTA for the indicated times, and then collected and disrupted by sonication in lysis buffer (25 mM Tris (pH 7.8), 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were standardized for β-gal activity. p47(phox) and Nrf2 translocation

HTSMCs were seeded in a 10-cm dish. After cells reached 90% confluence, they were shifted to DMEM/F-12 medium with 1% FBS for 24 h, and then incubated with 50 μg/ml LTA for the indicated times. The cells were washed once with ice-cold PBS, 300 μl of homogenization buffer A (20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 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 10 s at output 4 with a sonicator (Ultrasones) and centrifuged at 8000 rpm for 15 min at 4°C. The pellet (nuclear fraction) was resuspended in 300 μl of homogenization buffer B (1% Triton X-100 in buffer A) and sonicated for 10 s. The supernatant was...
centrifuged at 14,000 rpm for 60 min at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction). The membrane fraction was resuspended in 80 μl of homogenization buffer A. Samples from these supernatant fractions were denatured, subjected to SDS-PAGE, and transferred to nitrocellulose membrane. The translocation of p47phox or Nrf2 was identified by Western blot analysis using an anti-p47phox or anti-Nrf2 Ab, respectively.

Coimmunoprecipitation assay

Cell lysates containing 1 mg of protein were incubated with 2 μg of anti-TLR2 or anti-c-Src Ab at 4°C for 24 h, and then 10 μl of 50% protein A-Sepharose beads were added and incubated for 2 h at 4°C. The beads were washed extensively with homogenization buffer A and resuspended in 20 μl of 2× SDS-PAGE buffer. The samples were subjected to SDS-PAGE and Western blot analysis as described above.

FIGURE 1. Induction of HO-1 protein levels, mRNA expression, and promoter activity by LTA in HTSMCs. A, Cells were incubated with varying concentrations of LTA (0.5, 5, or 50 μg/ml) for the indicated times and harvested for the Western blot analysis of HO-1 protein expression. The same membranes were probed again with anti-GAPDH Ab to ensure equal loading of cellular proteins on the gel. Data are summarized from the time course study and expressed as mean ± SEM of three independent experiments. B, Cells exposed to 50 μg/ml LTA were harvested at the indicated times and total RNA was prepared. The RNA samples were analyzed by RT-PCR for the levels of HO-1 mRNA as described under Methods. The intensity of PCR product bands shown was quantitated by scanning densitometry and standardized to equivalent β-actin mRNA levels. C, Cells were transiently transfected with HO-1-luc reporter gene, and then treated with 50 μg/ml LTA for various times. The luciferase activity derived from HO-1 activation was normalized to the transfection efficiency with β-gal. Data represent mean ± SEM from at least three independent experiments. *, p < 0.05; #, p < 0.01 as compared with the basal level.
A-agarose beads was added and mixed for 24 h at 4°C. The immunoprecipitates were collected and washed three times with a lysis buffer without Triton X-100. Following wash, 5X Laemmli buffer was added and subjected to electrophoresis on 12% SDS-PAGE, and then blotted using an anti-TLR2, anti-MyD88, anti-TRAF6, anti-c-Src, or anti-p47phox Ab.

**Immunofluorescence staining**

HTSMCs were plated on 6-well culture plates with coverslips. Cells were shifted to DMEM/F-12 containing 1% FBS for 24 h, and then treated with 50 µg/ml LTA. When inhibitors were used, they were added 1 h before the application of LTA. After washing twice with ice-cold PBS, cells were fixed, permeabilized, and stained using an anti-Nrf2 Ab as previously described (22). The images observed under a fluorescent microscope (Axiovert 200M; Zeiss).

**Measurement of intracellular ROS accumulation**

The fluorescent probe DCF-DA was used to monitor net intracellular accumulation of ROS. This method is based on the oxidative conversion of nonfluorescent DCFH-DA (2',7'-dichlorohydrofluorescein diacetate) to fluorescent DCF by H2O2. HTSMCs were washed with warm HBSS and incubated in HBSS or cell medium containing 10 µM DCFH-DA at 37°C for 45 min. Subsequently, HBSS or cell medium containing DCFH-DA was removed and replaced with fresh cell medium. HTSMCs were then incubated with various concentrations of LTA. The fluorescent intensity (measured in relative fluorescence units) was at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (Appliskan; Thermo Scientific).

**Analysis**

Concentration-effect curves were fitted and EC50 values were estimated using the GraphPad Prizm program. Data were expressed as mean ± SEM and analyzed by one-way ANOVA followed with Tukey's posthoc test. A value of p < 0.05 was considered significant.

**Results**

**LTA induces HO-1 protein levels, mRNA expression, and promoter activity in HTSMCs**

To determine the effect of LTA treatment on HO-1 expression, cells were treated with various concentrations of LTA (0.5, 5, or 50 µg/ml) for 2, 4, 6, 16, or 24 h. The protein expression of HO-1 was determined by Western blot analysis. As shown in Fig. 1A, LTA induced HO-1 protein expression in a concentration- and time-dependent manner. LTA-induced HO-1 expression was significantly increased within 2 h and continued to increase over 24 h. To further examine whether the effect of LTA on HO-1 expression involved at the level of transcription, HO-1 mRNA was determined by RT-PCR. As shown in Fig. 1B, LTA (50 µg/ml) also induced HO-1 mRNA accumulation in a time-dependent manner with a maximal response within 4–5 h. Moreover, exposure to LTA also increased HO-1 promoter activity in a time-dependent fashion and peaked within 2–4 h (Fig. 1C).

**LTA-induced HO-1 expression requires ongoing transcription and translation**

To further determine whether LTA-induced HO-1 expression required transcription or translation, HTSMCs were stimulated with LTA (50 µg/ml) in the absence or presence of a transcriptional level inhibitor, actinomycin D or a translational level inhibitor, cycloheximide and HO-1 protein expression was determined by

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**FIGURE 3.** TLR2 and MyD88 are essential for the expression of HO-1 induced by LTA in HTSMCs. A. Cells were preincubated with anti-TLR2 or anti-TLR4 Ab (5 µg/ml) for 1 h, and then stimulated with 50 µg/ml LTA for 24 h. The cell lysates were subjected to Western blot analysis. Cells were transfected with a dominant negative mutant of TLR2 or MyD88, and then treated with 50 µg/ml LTA for 24 (B) or 5 h (C). B, The expression of TLR2, MyD88, and HO-1 were determined by Western blot analysis. C, The RNA samples were analyzed by RT-PCR for the levels of HO-1 mRNA. D, Cells were cotransfected with a dominant negative mutant of TLR2 or MyD88 and HO-1-luc reporter gene, and then treated with 50 µg/ml LTA for 4 h. The luciferase activity derived from HO-1 activation was normalized to the transfection efficiency with β-gal. Data represent the mean ± SEM from at least three independent experiments. *, p < 0.05 significant difference between compared groups. pcDNA was used as a control vector.
Western blot analysis. As shown in Fig. 2, LTA-mediated induction of HO-1 expression was abolished by either actinomycin D or cycloheximide in a concentration-dependent manner. Taken together, these findings demonstrate that the induction of HO-1 by LTA depends on de novo protein synthesis in HTSMCs.

Requirement of TLR2 and MyD88 for LTA-induced HO-1 expression in HTSMCs

TLRs play an important role for the host to detect and recognize the pathogens and initiate a rapid defensive mechanism (23). We have previously demonstrated that both TLR2 and TLR4 are expressed in HTSMCs (21). To investigate whether the LTA-induced HO-1 expression was mediated through these TLRs, HTSMCs were pretreated with anti-TLR2 or anti-TLR4 Ab (5 μg/ml) for 1 h, and then stimulated with 50 μg/ml LTA for 24 h. The expression of HO-1 was examined by Western blot analysis. A, Cells were pretreated with or without PP1 (10 μM) for 1 h, and then stimulated with 50 μg/ml LTA for various times. The cell lysates were subjected to Western blot analysis using anti-phospho-c-Src Ab at Tyr416.

Data are expressed as mean ± SEM of three independent experiments. *, p < 0.05 and **, p < 0.01 as compared with the basal level. ***, p < 0.05 and ###, p < 0.01 as compared with the cells exposed to LTA alone. Cells were transfected with Src siRNA or scrambled siRNA, and then treated with 50 μg/ml LTA for 24 (C) or 5 h (D). The expression of HO-1 was examined by Western blot analysis. D, The RNA samples were analyzed by RT-PCR for the levels of HO-1 mRNA. E, Cells were cotransfected with Src siRNA or scrambled siRNA and HO-1-luc reporter gene, and then treated with 50 μg/ml LTA for 4 h. The luciferase activity derived from HO-1 activation was normalized to the transfection efficiency with β-gal. Data represent mean ± SEM from at least three independent experiments. *, p < 0.05 for significant difference between compared groups.
FIGURE 5. LTA-induced HO-1 expression triggered by NADPH oxidase activation and ROS generation. A, Cells were pretreated with DPI, APO, or NAc, and then stimulated with 50 μg/ml LTA for 24 h. The expression of HO-1 was examined by Western blot analysis. B, Cells were pretreated with DPI (10 μM), APO (100 μM), or NAc (10 mM), and then stimulated with LTA (50 μg/ml) for 5 h. The levels of HO-1 mRNA were examined by RT-PCR. C, Cells were transiently transfected with HO-1-luc reporter gene, and then pretreated with DPI, APO, or NAc. After
HO-1 expression induced by LTA (Fig. 3B). In addition, MyD88 is an adaptor protein that is shared by all TLR pathways. To determine whether the induction of HO-1 occurred through MyD88, HTSMCs were transfected with a dominant negative mutant of MyD88, and then incubated with 50 \( \mu \text{g/ml} \) LTA for 24 h. As shown in Fig. 3B, transfection of HTSMCs with a dominant negative mutant of MyD88 markedly inhibited HO-1 expression induced by LTA. This down-regulation of HO-1 protein expression was further confirmed by reduction of HO-1 mRNA and promoter activity in HTSMCs transfected with dominant negative mutants of MyD88 and TLR2 (Fig. 3, C and D). These results suggest that LTA-induced HO-1 expression is mediated through the MyD88-dependent TLR2 signaling pathway in HTSMCs.

c-Src involves in LTA-induced HO-1 expression in HTSMCs

c-Src has been known to be recruited in the bacteria-induced TLR2 signaling leading to NF-\( \kappa \)B activation (24, 25). Therefore, we investigated the role of c-Src in LTA-mediated HO-1 induction. As shown in Fig. 4A, pretreatment with a c-Src kinase inhibitor PP1 attenuated LTA-induced HO-1 expression in a concentration-dependent manner. These results suggest that c-Src activation is involved in LTA-induced HO-1 expression in HTSMCs.

Regulation of c-Src activation occurs as a result of multiple phosphorylation sites on specific residues, including Tyr\( ^{416} \) and Tyr\( ^{527} \) (26). The major phosphorylation site of c-Src at the Tyr\( ^{416} \) residue results in activation from c-Src autophosphorylation (26). Thus, phosphorylation of c-Src at Tyr\( ^{416} \) is an important step in c-Src activation. We further examined c-Src phosphorylation at Tyr\( ^{416} \) stimulated by LTA in HTSMCs using an anti-phospho-c-Src Ab at Tyr\( ^{416} \). As shown in Fig. 4B, LTA stimulated a time-dependent Tyr\( ^{416} \) phosphorylation of c-Src with a maximal response within 5 min in HTSMCs. Moreover, pretreatment of HTSMCs with PP1 (10 \( \mu \text{M} \)) significantly attenuated c-Src phosphorylation in response to LTA during the period of observation. To further ensure that LTA-induced HO-1 expression was mediated via c-Src in HTSMCs, cells were transfected with Src siRNA. As shown in Fig. 4C, transfection with Src siRNA significantly down-regulated c-Src and subsequently led to a decrease of HO-1 expression.
FIGURE 8. LTA-enhanced NADPH oxidase activation is regulated by the formation of a TLR2/MyD88/TRAF6/c-Src/p47phox complex. A, Cells were transfected with a dominant negative mutant of TLR2, MyD88, or c-Src, and then treated with 50 μg/ml LTA for 10 min. The membrane and cytosolic fractions were prepared and subjected to Western blot analysis with anti-p47phox Ab. Gαs and GAPDH were used as marker proteins for membrane and cytosolic fractions, respectively. B, Cells were transfected with a dominant negative mutant of TLR2, MyD88, or c-Src, and then labeled with DCF-DA. After incubation for 45 min, cells were stimulated with LTA (50 μg/ml) for 1 h. The fluorescence intensity was measured. Data are expressed as mean ± SEM of three independent experiments. *, p < 0.05 and #, p < 0.01 for significant differences between compared groups. pcDNA was used as a control vector. C, Cells were stimulated for the indicated times with 50 μg/ml LTA. The cell lysates were subjected to immunoprecipitation with anti-TLR2 or anti-c-Src Ab, and then the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-TLR2, anti-MyD88, anti-TRAF6, anti-c-Src, or anti-p47phox Ab. D, Cells were pretreated with or without PP1 (10 μM) for 1 h, and then incubated in the absence or presence of 50 μg/ml LTA. After incubation for 10 min, total cell lysates were subjected to immunoprecipitation with an anti-p47phox Ab, and then the immunoprecipitates were analyzed by Western blot analysis using an anti-phosphotyrosine Ab. E, Cells were transfected with siRNA of MyD88, TRAF6, Src, or scrambled, and then treated with 50 μg/ml LTA for 5 min. The cell lysates were subjected to Western blot analysis using an anti-phospho-c-Src Ab at Tyr416. F, Cells were transfected with siRNA of MyD88, TRAF6, Src, or scrambled, and then incubated in the presence of LTA. The cell lysates were subjected to immunoprecipitation using an anti-TLR2 Ab, and then the immunoprecipitates were analyzed by Western blot analysis using anti-TLR2, anti-MyD88, anti-TRAF6, anti-c-Src, or anti-p47phox Ab.
protein expression in response to LTA. This down-regulation of HO-1 protein expression resulted from a reduction of HO-1 mRNA and promoter activity in HTSMCs transfected with Src siRNA (Fig. 4, D and E).

Involvement of NADPH oxidase and ROS generation in LTA-induced HO-1 expression

Several lines of evidence have demonstrated that ROS contributes to HO-1 expression in various cell types (15, 27, 28). 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 HO-1 expression in response to LTA was investigated. As shown in Fig. 5A, pretreatment of HTSMCs with NADPH oxidase inhibitors (DPI or APO) and a ROS scavenger NAc significantly abrogated LTA-induced HO-1 protein expression in a concentration-dependent manner. Moreover, pretreatment with DPI, APO, or NAc also inhibited HO-1 mRNA expression and promoter activity induced by LTA (Fig. 5, B and C).

Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of p47phox, p67phox, and p40phox. Phosphorylation of p47phox leads to a conformational change allowing its interaction with p22phox (29). It has been demonstrated that p47phox organizes the translocation of other cytosolic factors, hence its designation as “organizer subunit” (30). Therefore, we next investigated the effect of LTA on translocation of p47phox in HTSMCs. Cells were treated with 50 μg/ml LTA for the indicated times. The membrane and cytosolic fractions were prepared and subjected to Western blot analysis using an anti-p47phox Ab. As shown in Fig. 6A, LTA stimulated a time-dependent increase in translocation of p47phox from the cytosol to the membrane with a maximal response within 30 min. DPI (a flavoprotein inhibitor) and APO (an inhibitor of translocation of the oxidase subunits) have been shown to prevent p47phox translocation to the membrane (31, 32). This effect is confirmed by our observation that the LTA-stimulated p47phox translocation was attenuated by pretreatment with DPI or APO (Fig. 6B). To further ascertain that generation of ROS was involved in LTA-induced HO-1 expression in HTSMCs, a fluorescent probe DCF-DA was used to determine the generation of ROS in these cells. Cells were labeled with DCF-DA, treated with 50 μg/ml LTA for the indicated times, and the fluorescence intensity (relative DCF fluorescence) was measured at 485 nm excitation and 530 nm emission. As illustrated in Fig. 7A, LTA induced a significant increase in ROS levels within 5 min, peaked within 45 min, and sustained over 60 min. LTA-induced ROS generation was inhibited by pretreatment with NAc, DPI, or APO (Fig. 7B). These results indicate that NADPH oxidase activation and ROS generation play critical roles in LTA-induced HO-1 expression.

LTA-induced NADPH oxidase activation in HTSMCs is mediated by the formation of a TLR2/MyD88/TRAF6/c-Src/p47phox complex

The Src family kinases have been shown to mediate NADPH oxidase activation and ROS generation (18). Therefore, we investigated whether TLR2, MyD88, and c-Src involved in translocation of p47phox and production of ROS. As shown in Fig. 8A, LTA-induced p47phox translocation was abolished by transfection with dominant negative mutants of TLR2, MyD88, and c-Src. This suppressive effect on p47phox translocation was associated with a decrease in ROS generation stimulated by LTA (Fig. 8B). These results demonstrated that LTA induced ROS production via TLR2, MyD88, c-Src, and p47phox. Moreover, MyD88 and TRAF6 play key roles in TLR signaling. IL-1 has been shown to induce association of TRAF6 and c-Src (33). Thus, we further investigated the physical association of TLR2, MyD88, TRAF6, c-Src, and p47phox in LTA-induced NADPH oxidase activation. As shown in Fig. 8C, cells were transfected with siRNA of Nrf2 or scrambled, and then treated with 50 μg/ml LTA for 24 (A) or 5 h (B). The expression of HO-1 was examined by Western blot analysis. B. The RNA samples were analyzed by RT-PCR for the levels of HO-1 mRNA. C. Cells were cotransfected with siRNA of Nrf2 or scrambled and HO-1-luc reporter gene, and then treated with 50 μg/ml LTA for 4 h. The luciferase activity derived from HO-1 activation was normalized to the transfection efficiency with β-gal. Data represent the mean ± SEM from at least three independent experiments. *, p < 0.05 for significant difference between compared groups.
tyrosine phosphorylation of p47
1 h, and then stimulated with 50
orescence staining. Cells were pretreated with PP1, DPI, APO, or NAc for
GAPDH were used as a marker protein for nuclear and cytosolic fractions,
and subjected to Western blot analysis using an anti-Nrf2 Ab. Lamin A and
translocation, we further demonstrated the protein-protein interactions
C
B
A

LTA-induced HO-1 up-regulation is mediated via Nrf2 signaling
Nrf2 is a redox-sensitive basic leucine zipper transcription factor of NADPH oxidase complex that is activated by oxidative stresses and translocates into the nucleus. Activation of Nrf2 has been reported to play an important role in the ARE-driven expression of several detoxifying and antioxidant enzymes, including HO-1 (34). To determine the role of Nrf2 in LTA-mediated HO-1 induction, cells were transiently transfected with Nrf2 siRNA. As illustrated in Fig. 9A, transfection with Nrf2 siRNA down-regulated the protein expression of Nrf2 and subsequently decreased HO-1 expression induced by LTA. This suppressive effect was further supported by reduction of HO-1 mRNA and promoter activity stimulated by LTA (Fig. 9, B and C).

Moreover, translocation of Nrf2 between the cytosolic and nuclear fractions in LTA-treated HTSMCs was determined by Western blot analysis using an anti-Nrf2 Ab. Fig. 10A shows LTA-stimulated translocation from the cytosol into the nucleus in a time-dependent manner. The response was significantly elevated within 30 min, peaked at 2 h, sustained up to 4 h, and declined to the basal level within 6 h. The enhancement of Nrf2 translocation was attenuated by pretreatment with PP1, DPI, APO, or NAc (Fig. 10, B and C). To further ensure the translocation of Nrf2, we also observed nuclear translocation of Nrf2 by immunofluorescence staining. As illustrated in Fig. 10D, LTA-stimulated translocation of Nrf2 was inhibited by pretreatment with PP1, DPI, APO, or NAc. These results indicate that LTA-induced HO-1 expression was mediated through the c-Src/NADPH oxidase/Nrf2 pathway in HTSMCs.

Discussion
LTA has been shown to play an important role in the regulation of many gene expressions involved in many lung inflammatory diseases (35). Recently, HO-1 has been reported to be induced in airways of patients with asthma and chronic obstructive pulmonary disease (17). Moreover, overexpression of HO-1 has been shown to decrease airway inflammation, mucus secretion, and airway responsiveness to histamine in OVA-sensitized guinea pigs (36), suggesting that HO-1 plays a critical role in protecting the host during airway inflammation. Thus, the induction of HO-1 expression by various stress stimuli, such as LPS and oxidants, is thought to be an adaptive mechanism that protects the cells from oxidative injury (13). LPS has been shown to activate several intracellular signaling pathways, including PI3K/Akt, protein kinase C, or MAPK in various cell types. These signaling pathways in turn activate a variety of transcription factors, such as AP-1, NF-κB, or
Nrf2, which are implicated in the induction of HO-1 (13, 37–39). LTA can exert as LPS as an immune system-stimulating agent and plays a key role in the pathogenesis of severe inflammatory responses. However, the molecular mechanisms underlying LTA-induced HO-1 expression in HTSMCs remain unclear. In this study, the results demonstrated that the levels of HO-1 protein, mRNA, and promoter activity were increased upon stimulation with LTA. By Western blot, RT-PCR, and promoter activity analyses coupling with pharmacological inhibitors and transfection with dominant negative mutants or siRNAs revealed that LTA-induced HO-1 expression was mediated through a TLR2/MyD88/ TRAF6/c-Src/NADPH oxidase signaling pathway in HTSMCs. Moreover, the signals relayed into the nucleus that led to activation of transcription factors such as Nrf2 were determined by Western blot analysis and immunofluorescence staining. These results showed that ROS-dependent Nrf2 activation plays an important role in LTA-mediated HO-1 expression. Furthermore, TLR2, MyD88, TRAF6, and c-Src played as key roles in regulating LTA-mediated ROS generation through the activation of NADPH oxidase in HTSMCs.

During bacterial infection, the mammalian innate immune system can recognize bacteria and their cell wall components through two distinct receptors, CD14 and TLRs that initiate inflammatory responses (21). TLR2 acts as the receptor for LTA. A recent study has clearly shown that repurified LTA from S. aureus induces TNF-α secretion through the TLR2 signaling pathway (5). TLR2 has also been shown to contribute to HO-1 expression in traumatic brain injury (16). In our study, we found that LTA-mediated induction of HO-1 expression was inhibited by pretreatment with anti-TLR2 Ab, but not by anti-TLR4 Ab (Fig. 3A), consistent with the results that LTA induced activation of signaling transduction pathways mediated through TLR2 in various cell types (21, 40). This hypothesis was further supported by the findings that LTA induced HO-1 expression was attenuated by transfection with a dominant negative mutant of TLR2 (Fig. 3, B–D). Moreover, the TLR-regulated intracellular signaling is initiated by Toll/IL-1R domain-dependent heterophilic interactions with Toll/IL-1R domain-containing cytosolic adapters, such as MyD88 (4). Upon activation of TLR, MyD88 is recruited to TLR domains and links TLR with the downstream intracellular signaling cascades (41). This response is confirmed by our observation that LTA-enhanced HO-1 protein levels, mRNA expression, and promoter activity were attenuated by transfection with a dominant negative mutant of MyD88 (Fig. 3, B–D). c-Src is a member of the highly conserved Src family of protein tyrosine kinases, which consists of nonreceptor tyrosine kinases that display different expression patterns and have been implicated in numerous cellular processes, such as innate immune response and signaling induced by cytokines, Ags, and growth factors (42). c-Src has been known to be recruited in the bacteria-induced TLR2 signaling leading to NF-κB activation (24, 25). It also has been reported that Src is involved in LTA-mediated signalings in murine macrophages (3). In our study, LTA-stimulated HO-1 expression was attenuated by pretreatment with PP1 or transfection with Src siRNA (Fig. 4), suggesting that c-Src is also involved in the expression of HO-1 induced by LTA in HTSMCs.

When airway cells and tissues are exposed to oxidative stress, increased levels of ROS can have many deleterious effects within the airways. It has been shown that ROS can lead to impaired cellular functions and enhanced inflammatory reactions (43). Activated phagocytic cells produce large amounts of ROS. These cells are stimulated when encountering inhaled microorganisms or other mediators leading to the activation of NADPH oxidase and the production of superoxide anion. It has been reported that the expression of HO-1 due to NADPH oxidase activation and intracellular ROS generation induced by a range of stress stimuli, including LPS (44). Moreover, NAc is a thiol-containing compound that is used to decrease viscosity and elasticity of mucus. NAc is capable of scavenging hydrogen peroxide and hydroxyl radicals. Pretreatment of human alveolar and bronchial epithelial cells with NAc attenuates the injurious effects of hydrogen peroxide (45). In addition, DPI (a flavoprotein inhibitor) and APO (an inhibitor of translocation of the oxidase subunits) have been shown to prevent p47phox translocation to the membrane (31, 32). Therefore, we further investigated the roles of NADPH oxidase and ROS associated with HO-1 expression in response to LTA in HTSMCs, using NAc, DPI, and APO. In the present study, pretreatment with NAc, DPI, or APO inhibited LTA-induced HO-1 expression (Fig. 5). In addition, DPI and APO also retarded translocation of p47phox from the cytosol to the membrane (Fig. 6B) and intracellular ROS generation (Fig. 7B). These results showed that NADPH oxidase activation and ROS generation play key roles in LTA-induced HO-1 expression.

Although signaling pathways underlying LTA-regulated NADPH oxidase have not been completely defined, involvement of c-Src in NADPH oxidase activation and ROS generation has been reported (18). In this study, we also investigated the regulatory mechanisms involved in LTA-induced NADPH oxidase activation and ROS production. These results demonstrated that transfection with dominant negative mutants of TLR2, MyD88, and c-Src attenuated translocation of p47phox (Fig. 8A) and ROS generation (Fig. 8B), indicating the roles for TLR2, MyD88, and c-Src in LTA-induced p47phox translocation and ROS production.

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 (4). Signaling relayed between MyD88 and TRAF6 is mediated by members of the IL-1R-associated kinase family. In addition, IL-1 has been shown to induce association of TRAF6 and c-Src (33). Thus, we further investigated the physical association of TRAF6 and c-Src in LTA-induced NADPH oxidase activation. These results demonstrated that LTA induced the formation of a TLR2/MyD88/ TRAF6/c-Src/p47phox complex in HTSMCs (Fig. 8C). However, phosphorylation of p47phox at serine residues, in response to stimuli, has been demonstrated as a prerequisite for NADPH oxidase activation in phagocytic and nonphagocytic cells (46). As very little information is available on the role of tyrosine phosphorylation of p47phox and activation of NADPH oxidase, we therefore investigated whether LTA induced tyrosine phosphorylation of p47phox and the possible involvement of c-Src in tyrosine phosphorylation of p47phox. Our results suggested that LTA stimulated a Src-dependent tyrosine phosphorylation of p47phox (Fig. 8D). We further investigated the protein-protein interactions among TLR2, MyD88, TRAF6, c-Src, and p47phox. In this study, the results suggested that LTA stimulated Tyr416 phosphorylation of c-Src via TLR2, MyD88, and TRAF6. In addition, we demonstrated that Tyr416 phosphorylation of c-Src is not only critical for translocation of p47phox but also for the association among TLR2, MyD88, TRAF6, and p47phox (Fig. 8E). Although the detail protein-protein interactions among TLR2, MyD88, TRAF6, c-Src, and p47phox are not known, our results are the first time to show a novel role of TLR2/MyD88/TRAF6/c-Src/p47phox complex formation in LTA-induced NADPH oxidase activation and ROS production in HTSMCs. In the future, we will further determine which domains of TLR2, MyD88, TRAF6, c-Src, and p47phox are involved in protein-protein interactions caused by LTA.
More recently, Nrf2 has been shown to be a key factor in ARE-mediated gene induction of antioxidant proteins in response to various stimuli, including oxidative stress (19). Nrf2 is sequestered in the cytosol as an inactive complex with its repressor Keap1. Dissociation of Nrf2 from Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2. After forming a heterodimer with small Maf protein inside the nucleus, the active location and subsequent DNA binding of Nrf2. After forming a heterodimer with small Maf protein inside the nucleus, the active location and subsequent DNA binding of Nrf2. After forming a heterodimer with small Maf protein inside the nucleus, the active location and subsequent DNA binding of Nrf2.(20). Moreover, the deletion of the Nrf2 gene in mice resulted in a decrease in the basal levels of gene expression, including HO-1, GCL, and GST (34). In addition, LPS-induced HO-1 expression has been shown to be mediated via Nrf2 and protein kinase C in human monocytic cells (13). In the present study, transfection with Nrf2 siRNA almost completely inhibited LTA-induced HO-1 protein levels, mRNA expression, and promoter activity (Fig. 9). Furthermore, we also demonstrated that c-Src, NADPH oxidase, and ROS mediate LTA-induced Nrf2 translocation, using PP1, DPI, APO, and NAc (Fig. 10, B and C). Similarly, DPI and NAc have been reported to inhibit hyperoxia- and deltamethrin-induced Nrf2 nuclear translocation in pulmonary epithelial cells and PC12 cells, respectively (19, 47).

In summary, as depicted in Fig. 11, our results showed that in HTSMSCs, LTA induced ROS production through TLR2/MyD88/ TRAF6/c-Src/NADPH oxidase pathway to enhance ROS generation, which in turn initiates the activation of Nrf2 and ultimately induces HO-1 expression in HTSMSCs.

FIGURE 11. Schematic diagram illustrating the proposed signaling pathway involved in LTA-induced HO-1 expression in HTSMSCs. LTA activates the TLR2/MyD88/TRAF6/c-Src/NADPH oxidase pathway to enhance ROS generation, which in turn initiates the activation of Nrf2 and ultimately induces HO-1 expression in HTSMSCs.

References