Nontypeable *Haemophilus influenzae* induces COX-2 and PGE2 expression in lung epithelial cells via activation of p38 MAPK and NF-kappa B

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**Abstract**

**Background:** Nontypeable *Haemophilus influenzae* (NTHi) is an important respiratory pathogen implicated as an infectious trigger in chronic obstructive pulmonary disease, but its molecular interaction with human lung epithelial cells remains unclear. Herein, we tested the hypothesis that NTHi induces the expression of cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) via activation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kappa B in pulmonary alveolar epithelial cells.

**Methods:** Human alveolar epithelial A549 cells were infected with different concentrations of NTHi. The phosphorylation of p38 MAPK was detected by Western blot analysis, the DNA binding activity of NF-kappa B was assessed by electrophoretic mobility shift assay (EMSA), and the expressions of COX-1 and 2 mRNA and PGE2 protein were measured by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA), respectively. The roles of Toll-like receptor (TLR) 2 and TLR4, well known NTHi recognizing receptor in lung epithelial cell and gram-negative bacteria receptor, respectively, on the NTHi-induced COX-2 expression were investigated in the HEK293 cells overexpressing TLR2 and TLR4 in vitro and in the mouse model of NTHi-induced pneumonia by using TLR2 and TLR4 knock-out mice in vivo. In addition, the role of p38 MAPK and NF-kappa B on the NTHi-induced COX-2 and PGE2 expression was investigated by using their specific chemical inhibitors.

**Results:** NTHi induced COX-2 mRNA expression in a dose-dependent manner, but not COX-1 mRNA expression in A549 cells. The enhanced expression of PGE2 by NTHi infection was significantly decreased by pre-treatment of COX-2 specific inhibitor, but not by COX-1 inhibitor. NTHi induced COX-2 expression was mediated by TLR2 in the epithelial cell in vitro and in the lungs of mice in vivo. NTHi induced phosphorylation of p38 MAPK and up-regulated DNA binding activity of NF-kappa B. Moreover, the expressions of COX-2 and PGE2 were significantly inhibited by specific inhibitors of p38 MAPK and NF-kappa B. However, NTHi-induced DNA binding activity of NF-kappa B was not affected by the inhibition of p38 MAPK.

**Conclusion:** NTHi induces COX-2 and PGE2 expression in a p38 MAPK and NF-kappa B-dependent manner through TLR2 in lung epithelial cells in vitro and lung tissues in vivo. The full understanding of the role of endogenous anti-inflammatory PGE2 and its regulation will bring new insight to the resolution of inflammation in pulmonary bacterial infections.
Background
Nontypeable Haemophilus influenzae (NTHi) is one of common and important respiratory pathogens. NTHi causes otitis media and conductive hearing loss in children while pulmonary presence of this facultative intracellular pathogen is implicated as an infectious trigger in chronic obstructive pulmonary disease (COPD) in adults [1,2]. The emergence of antibiotic-resistance strains of NTHi and the difficulty of development of efficacious vaccines urge further efforts to understand the host response mechanisms involved in NTHi infections.

The respiratory epithelium is an important interface to environmental microorganisms. In addition to provide a physical barrier against microbial invasion and contribute to mucociliary clearance, respiratory epithelial cells are actively involved in inflammation and host defense of the lung in multiple ways. In particular, type 2 alveolar epithelial cells (AECs) as a defender of the alveolus are located in alveoli where they recognize invading pathogens by extracellular and intracellular receptors and contribute to host innate immunity [3-5]. Lipid metabolites of arachidonic acid such as prostaglandins have been shown to modulate immune and inflammatory responses [6,7]. Prostaglandin E2 (PGE2) is a product of the cyclooxygenase (COX) pathway. Two isoforms of COX, the constitutively expressed COX-1 and the inducible COX-2, have been identified. PGE2 is commonly thought to have proinflammatory effects on the pathogenesis of several inflammatory diseases including rheumatoid arthritis and periodontitis [7,8]. However, increasing evidence demonstrated that pulmonary PGE2 has a role in limiting the inflammatory response and tissue repair in contrast to its counterparts in other organs of the body [7].

The expression of COX-derived PGE2 and its molecular regulation depend on cell types and stimuli [9]. In the present study, we showed that NTHi induced COX-2 expression and subsequent PGE2 production via activation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kappa B in lung epithelial cells. The full understanding of the role of pulmonary endogenous anti-inflammatory mediators such as PGE2 and their regulation will bring new insight and develop novel treatment aiming at immune modulation.

Methods
Materials
SB203580, SB202190, PDTC, SC560, and NS398 were purchased from Sigma Chemicals (CA, USA), PGE2 ELISA kit was from R&D Co. (Minneapolis, USA). All other chemicals used were of analytical grade and obtained from commercial sources.

Isolation and identification of bacterial strain
NTHi strain was a clinical isolate from Second Affiliated Hospital of Medical School, Zhejiang University. The susceptible H. influenzae strains were confirmed by X, V and X+V factor requirement test, satellite test and API-NH identification system. Slide serum agglutination test was performed and the isolated strain was proved to not agglutinate with all the capsule antiserum of type a, b, c, d, e, and f. Finally, the isolated strain was identified by 16S rRNA gene amplification and sequencing. NTHi strain 12 was used for in vitro HEK239 cell experiments and in vivo mice experiments.

Mice experiments
C57BL/6 and BALB/c mouse strains, background strain for TLR2 and TLR4 knock-out, respectively, and TLR2 and TLR4 knock-out mice were used for NTHi-induced COX-2 expression in NTHi-induced pneumonia model in mice in vivo. C57BL/6 mice were purchased from National Cancer Institute (NCI, NIH, USA) and TLR2 knock-out mice were kindly provide by Dr. S. Akira, and TLR4 knock-out mice were purchased from Jackson Lab. (USA). Under the anesthesia, wild-type and TLR2 and TLR4 knock-out mice were intratracheally inoculated with 1 × 10⁷ CFU of NTHi strain 12. Mice lung tissues were collected 6 h after NTHi inoculation and mRNA expression of COX-2 was measured by quantitative RT-PCR (Q-PCR) as described below. All the animal experiments were approved by the Institutional Animal Care and Use Committee at University of Rochester.

Cell culture and in vitro experiments
A human alveolar epithelial cell line A549 (ATCC-CCL-185) was a kind gift from Shanghai ZJ Bio-Tech Co. Ltd., China. A549 cells were grown in 75 cm² polystyrene flasks with RPMI1640 (HyClone, Tauranga, New Zealand) supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY, USA). A549 cells were seeded at 1 × 10⁶ cells per well of 6-well flat-bottom, cell culture plates (Corning, NY, USA). This produced a confluent monolayer after overnight incubation at 37 °C in a 5% CO₂ humidified atmosphere. After growth medium was replaced by an antibiotic-free medium, A549 cells (1 × 10⁶ cells/mL) were infected with NTHi (multiplicity of infection, MOI: 1, 10, 50). Inhibition experiments were carried out by 1 h pretreatment with the p38 MAPK inhibitor SB203580 (20 µM), SB202190 (10 µM), the NF-kappa B inhibitor PDTC (40 µM) or the selective COX-1 inhibitor SC560 (5 µM), COX-2 inhibitor NS398 (10 µM) before bacterial stimulation. Supernatants after incubation were collected and stored at -70°C freezer for ELISA detection. HEK293 cells, stably overexpressing pcDNA, TLR2, and TLR4, were kindly provided by Dr. D.T. Golenbock, and cells were maintained in Dulbecco’s modified Eagle’s medium sup-
plemented with 10% FBS, 0.5 mg/mL of G418, and 10 µg/mL of ciprofloxacin (Cellgro, Herndon, VA).

Reverse transcription–polymerase chain reaction (RT-PCR)
Total RNA was isolated from A549 cells with Trizol Reagent (Invitrogen, CA, USA). For cDNA synthesis, 20 µL RT mixture containing total RNA 2 µg, dNTP 1 mM/L, Olig(dt)17, prime 0.2 µg, RNasin 20 U, M-MLV reverse transcriptase 200 U was incubated at 42°C for 60 min, then the reverse transcriptase was inactivated at 72°C for 15 min. PCR amplification was performed on a PE2400 cycler (Perkin-Elmer, Massachusetts, USA). Omiga 2.0 software (Oxford Molecular, Oxford, UK) was employed to design oligonucleotide primers specific for human COX-1, COX-2 and GAPDH (an internal control). COX-1: forward: 5’ AGTACCAGCAAGGTTTGGC 3', reverse: 5’ GCCGTCCTTGAATGTTAACG 3'; COX-2: forward: 5’ GACAGTCCCACTATTTCAAT 3', reverse: 5’ CATTCTTCATCAATTATCTGAT 3'; GAPDH: forward: 5' GACAGTCCCACTATTTCAAT 3', reverse: 5' ACTCCAGGACGTACCTAAGC 3'; COX-2: forward: 5' GACGTACTCAGC 3', with the product sizes 292 bp, 411 bp and 276 bp, respectively. The condition of PCR reactions was used as follows: 94°C for 5 min, then 94°C for 1 min; 63°C for COX-1, 56°C for COX-2 and 57°C for GAPDH for 1 min; 63°C for COX-1, 56°C for COX-2 and 57°C for GAPDH for 45 s for 30 cycles (GAPDH) or 40 cycles (COX-1, 2) and 72°C for 10 min to end the reaction. PCR products were electrophoresed by 1.5% agarose gel containing ethidium bromide.

Quantitative reverse transcription–polymerase chain reaction (Q-PCR)
Total RNA was isolated by using Trizol Reagent (Invitrogen), and the reverse transcription reaction was conducted with TaqMan reverse transcription reagents (Applied Biosystems). PCR amplifications were performed by using SYBR Green universal master mix for human and mouse COX-2. Reactions were amplified and quantified by using as ABI 7500 sequence detector. Relative quantity of mRNAs were obtained by using the comparative Ct method and was normalized by using TaqMan predeveloped assay reagent human cyclophilin and mouse GAPDH for human COX-2 and mouse COX-2, respectively. The primers for human COX-2 were as follows: forward: 5’GGTCCGCTTTAGCTTGTGCG 3' and reverse: 5’TTGCTCTATCTCTTGTTGAGCTTTGC 3'. The primers for mouse COX-2 were as follows: forward: 5’CCAGCCACTTCACCCATCTGGT 3' and reverse: 5’ACCCAGGCTTCCGTCTATGGA 3'.

Western blot
A549 were harvested by scrapers after stimulation of 15 min and 30 min. The cell pellets were lysed in lysis buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.5% bromophenol blue) and heated for 5 min at 95°C. Electrophoresis was performed at 200 V for 1 h with 12% SDS-PAGE at room temperature. Proteins were transferred to nitrocellulose membrane at 75 V for 1.5 h by wet blot at 4°C in Mini-Protein (Bio-Rad, California, USA). The membrane was then blocked with 5% non-fat dried milk in T-TBS for 1 h, washed three times with T-TBS and incubated with phospho-p38 MAPK antibody (Cell Signaling, Bevery, USA). Autoradiography was performed with optimized exposure times. Accordingly, p38 MAPK (Cell Signaling) was detected simultaneously to confirm equal protein load.

Electrophoretic mobility shift assay (EMSA)
After stimulation of A549 cells, nuclear protein was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Biotin-labeled consensus NF-kappa B oligonucleotides were purchased from Invitrogen (Shanghai, China). The sequence of oligo was: 5’ ATGTTGACGGACTTTCCACCC 3’. Briefly, EMSA binding reactions were performed by incubating 5 µg of nuclear extract with the annealed oligos according to the manufacturer’s instructions (Lightshift EMSA Kit, Pierce). The reaction mixture was subjected to electrophoresis on a 5% native gel.

Enzyme linked immunosorbent assay (ELISA)
PGE2 level in cell supernatants was measured using commercially available kits (R&D Systems) according to the manufacturer’s protocol. The limit of PGE2 assay was 39–5000 pg/ml.

Statistical analysis
All data were presented as means ± SEM. One-way ANOVA was used for statistical analysis of the differences between the groups. A value of P < 0.05 was considered statistically significant.

Results
NTHi-induced COX-2 expression in A549 cells
A549 cells were inoculated with NTHi at a MOI between 1 and 50 for 4 h, and RT-PCR analysis performed for the mRNA expression. As shown in Fig. 1, NTHi inoculation dose-dependently induced expression of COX-2 mRNA, whereas had no effect on COX-1 mRNA in A549 cells.

PGE2 release from NTHi-stimulated A549 cells in a COX-2 dependent way
To investigate the role of NTHi-induced COX-2 expression on the PGE2 production, A549 cells, first, were inoculated with NTHi at a MOI between 1 and 50 for 16 h, and PGE2
NTHi (MOI) | 0 | 1 | 10 | 50
---|---|---|---|---
COX-1 | | | | |
COX-2 | | | | |
GAPDH | | | | |

Figure 1
The mRNA expression of COX-2 but not COX-1 induced by NTHi in a dose-dependent manner. A549 cells were inoculated with different concentrations of NTHi, as indicated in the figure, and mRNA expression of COX-1 and COX-2 was measured by RT-PCR analysis.

expression was measured using ELISA assay. Result demonstrated that PGE2 release from A549 cells was significantly increased by NTHi (Fig. 2). Furthermore, the pre-treatment of selective COX-2 inhibitor NS398 significantly decreased NTHi-induced PGE2 production to baseline levels, but pre-treatment of selective COX-1 inhibitor SC560 has no effect on NTHi-induced PGE2 release in lung epithelial cells A549. Taken together, these data indicate that NTHi-induced PGE2 production was mediated by COX-2 dependent pathway, but independently from COX-1 pathway.

NTHi-induced COX-2 expression mediated by TLR2 signaling pathway
Given the fact that TLR2 and TLR4 are of particular important in NTHi-induced signaling pathway and gram-negative bacteria-initiated signaling pathway in lung epithelial cells, respectively, we investigated the possible involvement of TLR2 and TLR4 in NTHi-induced COX-2 expression in vitro by using HEK293 cells overexpressing TLR2 or TLR4. As shown in Fig. 3A, NTHi-induced COX-2 expression was significantly enhanced by overexpressing TLR2, but not by overexpressing TLR4. To further investigate the role of TLR2 and TLR4 in NTHi-induced COX-2 expression in vivo, wild type, and TLR2 and TLR4 knock-out mice were intratracheally inoculated with $1 \times 10^7$ CFU of NTHi strain 12, and mRNA expression of COX-2 in lungs of infected mice were measured by Q-PCR analysis 6 h after inoculation. As shown in Fig. 3B, NTHi-induced COX-2 expression in lung tissues of wild-type mice, and NTHi-induced COX-2 expression was abolished by TLR2-deficiency in TLR2 knock-out mice, but not by TLR4-deficiency in TLR4 knock-out mice. Taken together, these data indicate that NTHi-induced COX-2 expression is mediated by TLR2-dependent pathway, but independently from TLR4.

p38 MAPK instantly activated by NTHi
To investigate the involvement of p38 MAPK signaling pathway, which is known as important component of TLR2-induced signaling pathway, NTHi-induced phosphorylation of p38 MAPK was, first, measured by using western blot analysis. As shown in Fig. 4A, the phosphorylation of p38 MAPK was induced within 15–30 min in A549 cells after NTHi inoculation, and the pre-treatment of the specific inhibitor of p38 MAPK SB203580 blocked p38 MAPK signaling activated by NTHi (Fig. 4B).

Up-regulation of NF-kappa B DNA binding activity after NTHi stimulation
Since NF-kappa B signaling pathway is known as a component of TLR2 signaling pathway and downstream of p38 signaling pathway, we investigated whether NTHi induces NF-kappa B activation in our system, and p38 MAPK is up-stream molecule of NTHi-induced NF-kappa B signaling pathway. EMSA analysis showed that NF-kappa B translocation and its DNA binding activity were markedly increased by NTHi within 90 mins, and DNA binding activity of NF-kappa B was not affected by pre-treatment of specific p38 MAPK inhibitor SB203580. These data indicate that p38 MAPK and NF-kappa B are two independent signal pathways in the regulation of host response of human lung epithelium against NTHi (Fig. 5).
NTHi-induced COX-2 and PGE2 expression mediated by p38 MAPK and NF-kappa B signaling pathway

To determine the role of NTHi-induced p38 MAPK and NF-kappa B activations in NTHi-induced COX-2 and PGE2 expression, the effects of p38 MAPK inhibitors SB203580 and SB202190 and NF-kappa B inhibitor PDTC on the NTHi-induced COX-2 mRNA expression and PGE2 production were measured by using RT-PCR analysis and ELISA assay, respectively. As shown in Fig. 6A &6B, NTHi-induced COX-2 mRNA expression was markedly inhibited by pre-treatment of NF-kappa B inhibitor PDTC and p38 MAPK inhibitors SB203580 and
SB202190. Moreover, ELISA assay against PGE2 showed that NTHi-induced PGE2 expression was significantly inhibited by NF-kappa B inhibitor PDTC and p38 MAPK inhibitors SB203580 and SB202190. Taken together, these data clearly showed that NTHi-induced PGE2 expression is mediated by p38 MAPK- and NF-kappa B-dependent signaling pathway (Fig. 6C).

Discussion
Although NTHi is an important respiratory pathogen in both children and adults, little is known about its molecular interaction with human lung epithelial cells.

Equipped with transmembranous and cytosolic pattern recognition receptors, respiratory epithelium actively participates in immune reaction against invading pathogens instead of being only a passive physical barrier [10]. It has been shown that Streptococcus pneumoniae, Chlamydia pneumoniae, Moraxella catarrhalis, and respiratory syncytial virus induced COX-2 expression in pulmonary epithe-
Lipid products of arachidonic acid including prostaglandins play important roles in pulmonary immune regulation. PGE2 released by lung cells is demonstrated as a potent endogenous anti-inflammatory mediator, which modulates host immune response [7]. But, data about the molecular mechanisms of pulmonary PGE2 expression are very limited. In the present study, we demonstrated that NTHi induced COX-2 mRNA but not COX-1 mRNA in a dose-dependent manner in A549 cells. Furthermore, NTHi-induced PGE2 release was significantly suppressed by COX-2 specific inhibitor, but not COX-1 inhibitor. These results indicated that PGE2 release depends on COX-2 activity, in line with the previous studies by Schmeck et al. and Rupp et al. [9,14].

TLRs are well known cell surface receptors recognizing invading microbes and initiating cellular signaling pathways against bacterial components. Among of 11 mammalian TLRs, TLR2 is recognized as an important receptor for NTHi-induced signaling pathway in lung epithelial cells. Taken advantages of stably overexpressing TLR2 and TLR4 cells and TLR2 and TLR4 knock-out mice, we showed here that NTHi-induced COX-2 expression was mediated by TLR2 signaling pathway both in vitro and in vivo, but independently from TLR4. These data are in line with previous report that TLR2 is of particular important receptor for NTHi components [15,16].

The lung epithelial cell signaling networks activated by NTHi have been partially elucidated in recent years. Among these signaling pathways, p38 MAPK and NF-kappa B are two key molecules which coordinate the induction of multiple genes encoding inflammatory mediators and are involved in host immune responses to NTHi infections [2]. Activation of p38 MAPK up-regulates inflammatory cytokines, mucin MUC5AC and down-regulates TLR2 expression while activation of NF-kappa B is two key molecules which coordinate the induction of multiple genes encoding inflammatory cytokines, mucin MUC5AC and down-regulates TLR2 expression while activation of NF-kappa B are two key molecules which coordinate the induction of multiple genes encoding inflammatory cytokines, mucin MUC5AC and down-regulates TLR2 expression while activation of NF-kappa B are two key molecules which coordinate the induction of multiple genes encoding inflammatory cytokines, mucin MUC5AC and down-regulates TLR2 expression while activation of NF-kappa B are two key molecules which coordinate the induction of multiple genes encoding inflammatory cytokines, mucin MUC5AC and down-regulates TLR2 expression while activation of NF-kappa B are two key molecules which coordinate the induction of multiple genes encoding inflammatory cytokines, mucin MUC5AC and 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Conclusion
In conclusion, NTHi induces COX-2 and PGE2 expression in a p38 MAPK and NF-kappa B-dependent manner through TLR2 in lung epithelial cells in vitro and lung tissues in vivo. The full understanding of the role of endogenous anti-inflammatory PGE2 and its regulation will bring new insight to the resolution of inflammation in pulmonary NTHi infections.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
FX planned the experimental design, carried out Western blot, EMSA and drafted the manuscript. ZQW carried out ELISA. LJH performed in vitro experiments. KT carried out Q-PCR. JDL participated in the study design and helped to draft the manuscript. HHS participated in the study design and coordinated the research group. All authors read and approved the final manuscript.

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