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Synergistic activation of NF-κB by nontypeable *H. influenzae* and *S. pneumoniae* is mediated by CK2, IKKβ-IκBα, and p38 MAPK

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Abstract

In review of the past studies on NF-κB regulation, most of them have focused on investigating how NF-κB is activated by a single inducer at a time. Given the fact that, in mixed bacterial infections *in vivo*, multiple inflammation inducers, including both nontypeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae*, are present simultaneously, a key issue that has yet to be addressed is whether NTHi and *S. pneumoniae* simultaneously activate NF-κB and the subsequent inflammatory response in a synergistic manner. Here, we show that NTHi and *S. pneumoniae* synergistically induce NF-κB-dependent inflammatory response via activation of multiple signaling pathways *in vitro* and *in vivo*. The classical IKKβ-IκBα and p38 MAPK pathways are involved in synergistic activation of NF-κB via two distinct mechanisms, p65 nuclear translocation-dependent and -independent mechanisms. Moreover, casein kinase 2 (CK2) is involved in synergistic induction of NF-κB via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. These studies bring new insights into the molecular mechanisms underlying the NF-κB-dependent inflammatory response in polymicrobial infections and may lead to development of novel therapeutic strategies for modulating inflammation in mixed infections for patients with otitis media and chronic obstructive pulmonary diseases.

Keywords: Synergistic activation; NF-κB; CK2; Polymicrobial infection

Gram-negative bacterium nontypeable *Haemophilus influenzae* (NTHi) and Gram-positive bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) are important human pathogens [1,2]. In children, they cause otitis media (OM), the most common childhood infection and the leading cause of conductive hearing loss [3], while in adults, they exacerbate chronic obstructive pulmonary diseases (COPD), the fourth leading cause of death in the United States [1,2]. Although a majority of OM or COPD is mainly associated with a single bacterial pathogen, there is a growing body of evidence that a portion of patients diagnosed with OM or COPD have mixed infections of NTHi and *S. pneumoniae* [4,5]. Like most other bacterial infections, both OM and COPD are also characterized by inflammation, which is mainly mediated by inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-8 (IL-8) [6,7]. Among a variety of transcription regulators, nuclear factor κB (NF-κB) has been shown to play a critical role in regulating the expression of large numbers of genes encoding cytokines, chemokines, and other mediators involved in inflammatory responses [8,9].

Over the past two decades, tremendous efforts have been made toward understanding how NF-κB is activated by a variety of inducers including bacteria, virus, and cytokines. However, in review of the past studies on NF-κB regulation, most of them have focused on investigating how

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NF-κB is activated by a single inducer at a time. Given the fact that, in mixed infections *in vivo*, multiple inflammation inducers, including both NTHi and *S. pneumoniae*, are present simultaneously, a key issue that has yet to be addressed is whether NTHi and *S. pneumoniae* simultaneously activate NF-κB and the subsequent inflammatory response in a synergistic manner.

In the present study, we report that NTHi and *S. pneumoniae* synergistically induce NF-κB-dependent inflammatory response via multiple signaling pathways *in vitro* and *in vivo*. The classical IKKβ-IκBα and MAPK p38 signaling pathways are involved in synergistic activation of NF-κB via p65 nuclear translocation-dependent and -independent mechanisms. Moreover, CK2 is involved in synergistic induction of NF-κB via a mechanism dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. These studies bring novel insights into the molecular mechanisms underlying the synergistic activation of NF-κB in polymicrobial infections and may help to identify novel therapeutic targets for treating patients with otitis media and chronic obstructive pulmonary diseases.

Materials and methods

Reagents. CK2 inhibitor, SB203580, and MG132 were purchased from Calbiochem (La Jolla, CA).

Bacterial strains and culture condition. NTHi strain 12 and *S. pneumoniae* strain 6B were used in this study. Bacteria were grown on chocolate agar at 37 °C in an atmosphere of 5% CO_2. NTHi crude extracts were used as described [10]. For making *S. pneumoniae* crude extracts, *S. pneumoniae* were harvested from a plate of chocolate agar after overnight incubation and incubated in 100 ml of Todd–Hewitt broth and yeast extracts. After overnight incubation, *S. pneumoniae* was centrifuged at 10,000*g* for 10 min, and the supernatant was discarded. The resulting pellet of *S. pneumoniae* was suspended in 10 ml of phosphate-buffered saline and sonicated. Subsequently, the lysates were collected and stored at −70 °C.

Cell culture. Human epithelial cell lines HeLa, A549, and HMEEC-1 and primary airway epithelial NHBE cells were maintained as described [7,10,11] and used for all experiments unless otherwise indicated. All mouse embryonic fibroblast (MEF) cells were maintained as described [12]. Wild type (WT), IKKα$^{-/-}$, and IKKβ$^{-/-}$ MEFs were kindly provided by Dr. I. Verma.

Real-time quantitative PCR analysis of TNF-α, IL-1β, and IL-8. TRIzol® Reagent (Invitrogen) by following the manufacturer's instruction. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37 °C, followed by 60 min at 42 °C by using oligo(dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix for human TNF-α, IL-1β, and IL-8 as described previously [12].

Plasmids, transfection, and luciferase activity assays. Expression plasmids IκBα (S32/36A), IKKα (K44M), IKKβ (K49A), fp38α (AF), and fp38β2 (AF) have been described previously [7,10]. The reporter construct NF-κB luc was generated as described [10]. It contains three copies of the NF-κB site from IL-2 receptor promoter by using following oligonucleotides: 5′-TCGAGACGGCAGGGGAATCTCCCTCTCCG-3′ and 3′-CTGCCGTCCCCTTAGAGGGAGAGGCAGCT-5′. All transient transfections were carried out in triplicate using a TransIT-LT1 reagent from Mirus (Madison, WI) following the manufacturer's instructions. At 40 h after starting the transfection, cells were pretreated with or without chemical inhibitors including CK2 inhibitor, MG132, and SB203580 for 1 h. NTHi or *S. pneumonia*e were then added to the cells for 5 h before cell lysis for luciferase assay. Luciferase activity was normalized with β-galactosidase activity.

Western blot analysis. Western blot analysis was performed as described [11]. Antibodies against phospho-p38, p38, phospho-IκBα, total IκBα, phospho-p65 (S536), and phospho-p65 (S276) were purchased from Cell Signaling Technology. Antibodies against p65 and TFIIB were purchased from Santa Cruz Biotechnology. Antibody against β-actin was purchased from Sigma. Antibody against CK2 was purchased from BD biosciences.

Immunofluorescent staining. Immunofluorescent staining was performed as described ([7,10], and Supplementary Material).

Nuclear protein extraction and electrophoretic mobility-shift assay (EMSA) and super shift assay. Nuclear protein extraction and EMSA were performed as described ([13] and Supplementary Material).

RNA-mediated interference. RNA-mediated interference for down-regulating CK2 expression was done using SiRNA-CK2β (Dharmacon) as described ([11,12], and Supplementary Material).

CK2 kinase assay. (Supplementary Material).

Mouse and animal experiments. BALB/c mice were purchased from Charles River Laboratories, and all animal experiments were approved by the Institutional Animal Care and Use Committee at House Ear Institutes and University of Rochester. Under the anesthesia, mice were intratracheally inoculated with *S. pneumoniae* (1.25×10^7 CFU), NTHi (3×10^7 CFU), or *S. pneumoniae* with NTHi for 3 h, saline was inoculated as control. Broncho-alveolar lavage (BAL) was performed by cannulating the trachea with sterilized PBS, and cells from BAL fluid were stained with Wright-Giemsa stain after cytocentrifuge. For cytokine mRNA expression analysis, total RNA was extracted from whole lung tissues of mice inoculated with *S. pneumoniae*, NTHi, or *S. pneumoniae* with NTHi for 3 h, and real-time quantitative PCR (Q-PCR) was performed as described above. For CK2 inhibition experiment *in vivo*, CKII inhibitor (5 mg/kg) was inoculated intraperitoneally 2 h prior to bacterial inoculation.

Results

NTHi synergizes with S. pneumoniae to induce NF-κB-dependent inflammatory response in vitro and in vivo

To determine whether Gram-negative bacterium NTHi synergizes with Gram-positive bacterium *S. pneumoniae* to induce NF-κB activation and NF-κB-dependent inflammatory response, we first assessed NF-κB-dependent transcriptional activity by using NF-κB-dependent luciferase reporter construct in human epithelial HeLa cells. As shown in Fig. 1A, NTHi and *S. pneumoniae* synergistically induced NF-κB-dependent promoter activity. Similar results were also observed in human airway epithelial cell line A549, middle ear cell line HMEEC-1, and human primary bronchial epithelial NHBE cells (data not shown), suggesting that synergistic activation of NF-κB by NTHi and *S. pneumoniae* may be generalizable to a variety of human epithelial cells. Consistent with this result, p65, the key subunit of NF-κB complex, was translocated into the nucleus 15 min after simultaneous treatment with NTHi and *S. pneumoniae*, as assessed by performing Western blot analysis using nuclear protein in HeLa cells (Fig. 1B). Similar result was also observed in HeLa cells by performing immunofluorescent staining using antibody against p65 (Supplementary Fig. 1A). Moreover, NTHi and *S. pneumoniae* also synergistically increased DNA binding activity of NF-κB as assessed by performing electrophoretic mobility shift assay (EMSA) (Fig. 1C). Further analysis by