Oxidative Stress Enhances Toll-Like Receptor 3 Response to Double-Stranded RNA in Airway Epithelial Cells

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Virus infections are a major cause of chronic obstructive pulmonary disease (COPD) exacerbations. Recently, Toll-like receptor 3 (TLR3) has been demonstrated to react to double-stranded RNA (dsRNA) and to be involved in the immune responses after viral infections. In the present study, we examined whether oxidative stress, which is involved in the pathogenesis of COPD, enhances the responses of TLR3 in airway epithelial cells. The effect of hydrogen peroxide (H_2O_2) on the release of IL-8 from BEAS-2B cells and primary human bronchial epithelial cells after stimulation with polyinosine-polycytidylic acid [poly(I:C)], a synthetic analog of viral dsRNA and a ligand for TLR3, and the signal transduction were examined. One hundred to 150 μM H_2O_2 significantly potentiated the release of IL-8 from the epithelial cells after stimulation with 10 μg/ml poly(I:C). The H_2O_2-augmented IL-8 release was inhibited by treatment with N-acetylcysteine. One hundred micromoles of H_2O_2 enhanced the translocation of nuclear factor (NF)-κB p65, but not that of interferon regulatory factor-3 (IRF-3), into the nucleus and the NF-κB DNA binding activity after poly(I:C) stimulation, which effect was inhibited not by the silencing of IRF-3 but by MG132, a proteasome inhibitor, or dexamethasone. One hundred micromoles of H_2O_2 potentiated the TLR3 expression on the airway epithelial cells treated with poly(I:C). These data suggest that oxidative stress augments the response of TLR3 in airway epithelial cells via NF-κB and that this effect might be partly mediated by the enhancement of TLR3 expression. Modulation of this pathway may be a therapeutic target for viral-induced exacerbations of COPD.

Keywords: chronic obstructive pulmonary disease; exacerbation; hydrogen peroxide; nuclear factor-κB

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the world, and further increases in its prevalence and mortality can be expected in the coming decades (1). Many patients with COPD experience periodical exacerbations, which are worsenings of their symptoms such as cough, sputum, and/or dyspnea, causing an acute deterioration in lung function and airway inflammation (1–4). Preventing the exacerbations is necessary to inhibit the progression of the disease and to reduce medical expenses for hospitalization during exacerbations (3–5). However, the precise mechanism of these exacerbations has not been fully elucidated.

In COPD, inflammatory cells including macrophages, neutrophils, and CD8-positive lymphocytes are involved in the pathophysiology of the airway inflammation. These cells are activated by cigarette smoke and release proteases including neutrophil elastase and matrix metalloproteinases, which break down connective tissue in the lung parenchyma and stimulate mucus hypersecretion (6, 7). Oxidants derived from cigarette smoke or released by inflammatory cells play a pivotal role in the pathogenesis of COPD (6) and, in fact, oxidative stress markers including hydrogen peroxide (H_2O_2) are elevated in the airways of patients with COPD (8–11). During exacerbations, greater amounts of H_2O_2 are produced in the airways of patients with COPD than those in the stable disease condition (12, 13). In addition, antioxidants N-acetylcysteine (NAC) and carbocysteine have been shown to reduce the number of COPD exacerbations (14, 15), suggesting that oxidative stress might be involved in the exacerbation of COPD.

CLINICAL RELEVANCE

Oxidative stress can augment the response of Toll-like receptor 3 (TLR3) in airway epithelial cells via NF-κB, and this effect might be partly mediated by the enhancement of TLR3 expression. Modulation of this pathway may be a therapeutic target for viral-induced exacerbations of chronic obstructive pulmonary disease.

Recently, viral infections including rhinovirus, corona virus, respiratory syncytial virus (RSV), and influenza virus have been demonstrated to be a major cause of COPD exacerbations (16, 17). In addition, the role of Toll-like receptors (TLRs) in recognizing the pathogen-associated molecular patterns, including TLR3, TLR7, and TLR8, has been demonstrated in innate immunity (18, 19). Especially, TLR3 reacts with viral-derived double-strand RNA (dsRNA) and is thought to play a key role in the viral infection in the respiratory system (20–22). The expression of TLR3 has been detected on airway epithelial cells, dendric cells, and macrophages (20, 22). When TLR3 is activated, both nuclear factor (NF)-κB and interferon regulatory factor-3 (IRF-3) are translocated into the nucleus and activate their DNA binding (18, 21, 23). The former has been reported to regulate the expression of proinflammatory cytokines, including TNF-α, IL-1β, and the potent neutrophil chemoattractant IL-8 (24). The latter regulates the expression of type I interferon (IFN) (23). Although both oxidative stress and viral infection are involved in the exacerbations in COPD, it has been not elucidated whether oxidative stress potentiates the TLR signaling and mediator production.

IL-8 is a potent neutrophil chemoattractant and is related to the accumulation of neutrophils in the airways of patients with COPD (25). In patients with COPD, the levels of IL-8 in sputum, bronchoalveolar lavage, exhaled breath condensate, and serum are elevated compared with healthy subjects (26–29), and the level is further increased during exacerbations (28, 30, 31), which leads to airway neutrophilia (32, 33). Airway epithelial cells produce large amounts of IL-8, and TLR3 activation induces IL-8 production in airway epithelial cells (21, 22).

The present study, therefore, was designed to determine, using a synthetic dsRNA, polyinosinic-cystidic acid [poly(I:C)], the following: (*1*) whether oxidative stress could affect the

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poly(I:C)-mediated IL-8 release in airway epithelial cells; (*2*) whether oxidative stress modulates the poly(I:C)-mediated TLR3 signaling; and (*3*) whether oxidative stress affects TLR3 expression on airway epithelial cells.

MATERIALS AND METHODS

Materials

Commercially available reagents were obtained as follows: R848 and bafilomycin were purchased from Alexis Biochemicals (Lausen, Switzerland); poly(I:C) was from Amersham Biosciences (Piscataway, NJ); MG132, a proteasome inhibitor, and R837 were from Calbiochem (La Jolla, CA); control rabbit IgG was purchased from Dako (Glostrup, Denmark); serum-free Keratinocyte Basal Medium and its supplement including recombinant epidermal growth factor and bovine pituitary extract, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Life Technologies (Grand Island, NY); 3′,5,5′-tetramethyl benzidine (TMB), propidium iodide (PI), H_2O_2, lipopolysaccharide (LPS), N-acetylcysteine (NAC), dexamethasone (DEX), Hanks' balanced salt solution (HBSS), RPMI 1640 medium, bovine serum albumin (BSA), paraformaldehyde, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, thiazolyl blue tetrazolium, and dimethyl sulphoxide (DMSO) were from Sigma Aldrich, Inc. (St. Louis, MO).

Preparation of Epithelial Cells

The human bronchial epithelial cell line (BEAS-2B cells) was obtained from the American Type Culture Collection (Rockville, MD). Four strains of primary human bronchial epithelial cells (HBEpC) were purchased from Cell Aplications, Inc. (San Diego, CA) and ScienCell research laboratories (Carlsbad, CA). BEAS-2B cells (passages 45–55) or HBEpC (passages 3–7) were cultured in serum-free Keratinocyte Basal Medium supplemented with 10 ng/ml recombinant epidermal growth factor and 30 μg/ml bovine pituitary extract. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and passaged. Cells were routinely grown to 80% confluence and growth arrested overnight before experimental procedures by transfer to growth factor–free media. Cells were cultured in 96-well plates for investigation of the effect of poly(I:C)-induced IL-8 release. To investigate the effect of poly(I:C)-induced IL-8 release, the supernatants were harvested at 24 hours after treatment with poly(I:C) and stored at −80°C until the measurement. To estimate the effect of H_2O_2 on the poly(I:C)-induced IL-8 release, H_2O_2 was added to the media 30 minutes before the treatment with poly(I:C) (34). To evaluate the effects of bafilomycin, NAC, MG132, and DEX, these drugs were added to the media at various concentrations 30 minutes before H_2O_2 or poly(I:C) treatment.

Detection of TLR3 by Immunocytochemistry

Cells were seeded in 8-well chamber slides at a density of 1×10^5/ml and cultured for 24 hours, and then the medium was replaced with growth factor–free media for a further 24 hours. After washing with PBS, the slides were fixed with freshly prepared 4% paraformaldehyde in PBS for 10 minutes at room temperature. The slides were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. The slides were blocked for 1 hour at room temperature by 5% BSA and then rinsed. The slides were then incubated overnight with goat polyclonal anti-TLR3 antibody (10 μg/ml; R&D Systems, Minneapolis, MN) at 4°C. After washing with PBS, the slides were incubated with FITC-conjugated rabbit anti-goat IgG antibodies (1: 2,000 dilution; Sigma) for 1 hour at room temperature. Nuclei of the cells were stained with PI (1 μg/ml). The slides were then viewed with an epifluorescence microscope (E-800; Nikon, Tokyo, Japan) and photographed with a digital camera (DMX-1200C; Nikon) under ×400 magnification.

Detection of TLR3 by Immunoblotting

Cells were treated with 10 μg/ml poly(I:C) in the presence or absence of 100 μM H_2O_2 for 24 hours. After washing with HBSS, cells were homogenized in cell lysis buffer (0.05% Triton X, 35 mM Tris-HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 μM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 1 μg/ml leupeptin) at 4°C. Samples were solubilized in SDS-PAGE sample buffer. Equal amounts of protein and recombinant human TLR3 (R&D Systems, Minneapolis, MN) for positive control were loaded and separated by electrophoresis on 12.5% SDS polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Goat polyclonal anti-TLR3 antibody (0.2 μg/ml; R&D Systems, Minneapolis, MN) or mouse monoclonal anti–β-actin antibody (1:10,000 dilution; Sigma) were used for the detection of target proteins. Peroxidase-conjugated appropriate secondary antibodies were used. Binding antibodies were detected using ECL-plus (Amersham Biosciences, Buckinghamshire, UK) and visualized with a chemiluminescence imaging system (Luminocapture AE6955; Atto Co., Tokyo, Japan). Each band intensity was quantified by densitometry (Image J; NIH, Frederick, MD).

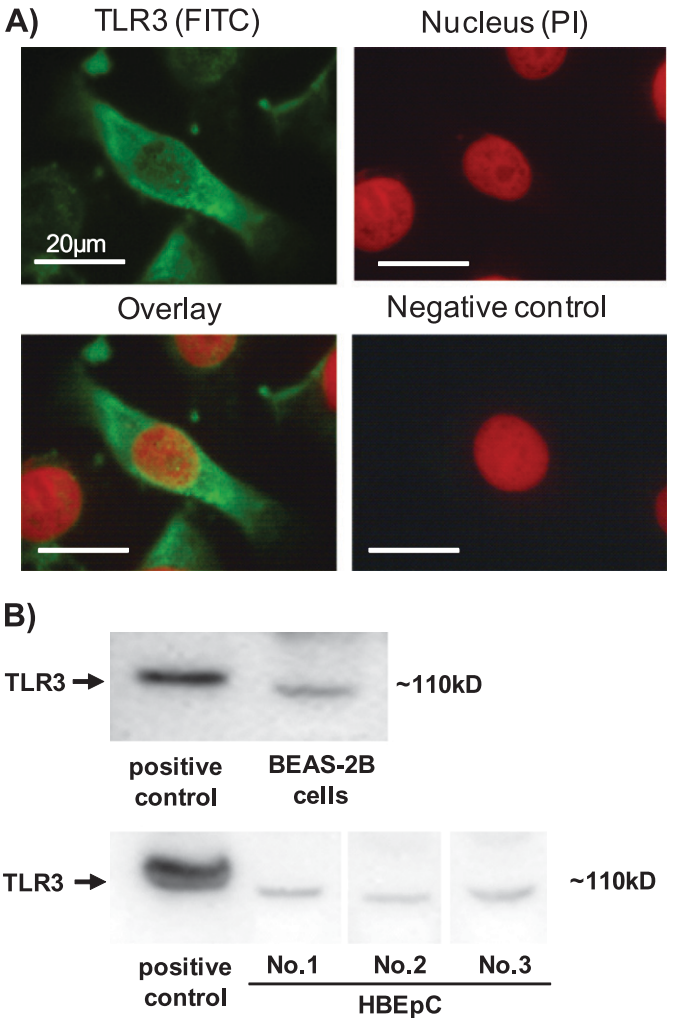

Figure 1. Expression of Toll-like receptor 3 (TLR3) in human bronchial epithelial cells. (*A*) Panels show representative photographs of immunoreactivity of TLR3 in BEAS-2B cells (*upper left panel*, *green*), immunofluorescence of the nucleus (*upper right panel*, *red*), an overlaid image (*lower left panel*), and a negative control (*lower right panel*). *Original magnification*: ×400. (*B*) The expression of TLR3 in BEAS-2B cells and three different strains of primary human bronchial epithelial cells was detected at 110 kD by immunoblotting. Recombinant human TLR3 was used for positive control. PI, propidium iodide; HBEpC, primary human bronchial epithelial cells.

Cell Viability Assay by MTT Assay Method

One milligram per milliliter of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was prepared using HBSS. Once the supernatants had been removed from the cells, 50 μl MTT solution

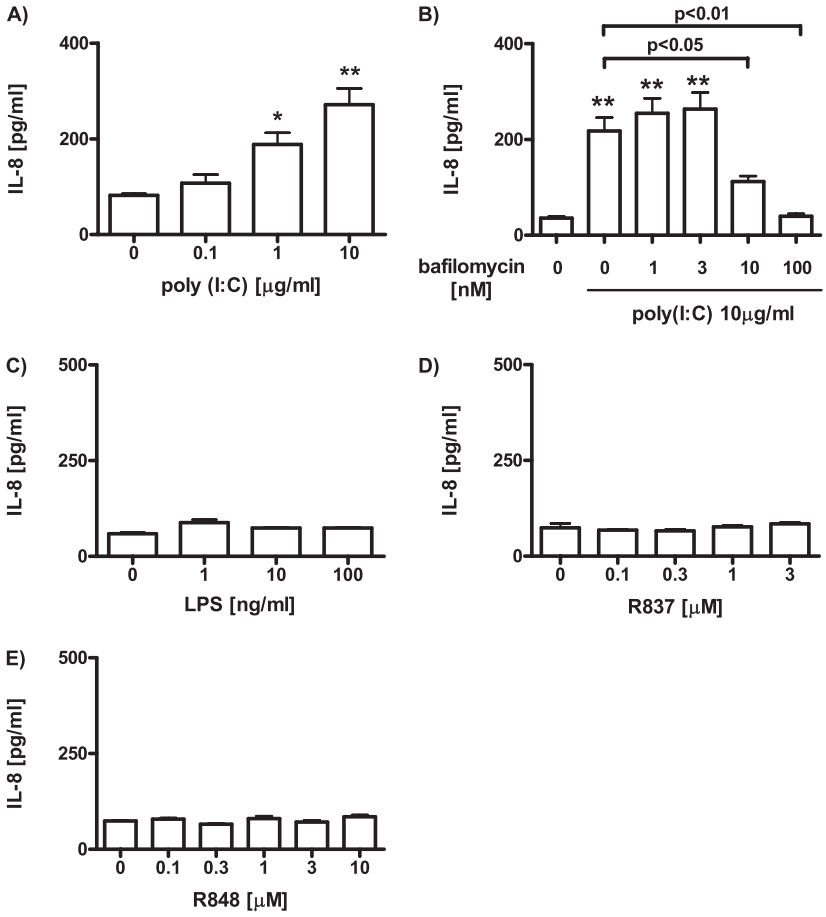

Figure 2. Effect of ligands for TLRs on IL-8 release and effect of bafilomycin on poly(I:C)-induced IL-8 release in BEAS-2B cells. (*A*) BEAS-2B cells were treated with various concentrations of poly(I:C). (*B*) The cells were treated with 10 μg/ml poly(I:C) or vehicle in the presence of various concentrations of bafilomycin, an inhibitor of TLR3 that blocks endosomal H^+-ATPase activity. After 24 hours, supernatants were harvested and assayed for IL-8 by enzyme-linked immunosorbent assay (ELISA). The data are expressed as mean values ± SEM for three to four separate experiments. $*P < 0.05$, $**P < 0.01$ compared with the values of control. (*C–E*) BEAS-2B cells were treated with various concentrations of (*C*) lipopolysaccharide (LPS), a ligand for TLR4; (*D*) R837, a ligand for TLR7; or (*E*) R848, a ligand for TLR7/8. After 24 hours, supernatants were harvested and assayed for IL-8 by ELISA.

were added to each well. The plates were incubated for 1 hour at 37°C, after which the MTT solution was discarded. Fifty microliters of DMSO were added to each well. The product was quantified at 550 nm with a microplate reader.

Measurement of IL-8 Using Enzyme-Linked Immunosorbent Assay

IL-8 was measured in the supernatants using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The lower detection limit was 16 pg/ml.

Detection of NF-κB (p65) and IRF-3 by Nuclear Extraction and Immunoblotting

Cells were treated with 10 μg/ml poly(I:C) in the presence or absence of 100 μM H_2O_2 for 0 to 120 minutes. After washing with HBSS, cells were homogenized in cell lysis buffer to obtain the nuclear fraction using Nuclear Extraction Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Samples were separated by electrophoresis and blotted on a PVDF membrane (Bio-Rad). The following antibodies were used for detection of the target proteins: mouse monoclonal anti–NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti–IRF-3 antibody (1:500 dilution; Santa Cruz Biotechnology), or mouse monoclonal anti-lamin A/C antibody (1:400 dilution; Santa Cruz Biotechnology). Peroxidase-conjugated appropriate secondary antibodies were used. The following detection and visualization procedures were performed the same as for TLR3 immunoblotting.

NF-κB p65 DNA Binding Activity

To assess NF-κB p65 DNA binding activity, nuclear fraction was obtained after 60 minutes of incubation with poly(I:C) or vehicle and evaluated by NF-κB p65 transcription factor assay kit, which is based on the method of ELISA (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. Briefly, equal amounts of nuclear extract were added on 96-well plates which immobilized a specific double-stranded DNA sequence containing the NF-κB response element on the bottom. After incubation and washing, the plates were incubated with anti-human NF-κB p65 primary antibody. Then a goat anti-rabbit HRP-conjugated secondary antibody was used. After developing, the plates were read at 450 nm with a microplate reader.

Knockdown of IRF-3 by siRNA

Cells were seeded in 6-well plates in complete media. At 50% confluence, transfection with siRNA was performed. In one tube, 40 μl of lipofectamine 2000 (Invitrogen Life Technologies) were mixed gently with 1.5 ml Opti-MEM medium (Invitrogen Life Technologies) and incubated for 5 minutes at room temperature. In a separate tube, 975 pmol of either nontargeting or IRF-3 siRNA (SMART pool plus; Dharmacon, Lafayette, CO) were mixed gently with 1.5 ml Opti-MEM medium. These siRNA and lipofectamine solutions were then combined, gently mixed, and incubated for 20 minutes at room temperature. After incubation, 2 ml of DMEM were added to obtain a final volume of 5 ml (final concentration of siRNAs = 200 nM), which was added to each dish. Cells were then incubated at 37°C for 6 hours. After that, media were changed to serum-free Keratinocyte Basal Medium with growth factor and further cultured for 24 hours. The siRNA-treated cells were then used to assess the role of the IRF-3 on IL-8 release in the presence of 10 μg/ml poly(I:C) or to assess IRF-3 expression by immunoblotting.

Statistical Analysis

Data were expressed as means ± SEM. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to perform all statistical tests. Experiments with multiple comparisons were evaluated by one-way ANOVA followed by Bonferroni's test to adjust for multiple comparisons. A paired two-group Student's *t* test was used for single comparisons. Probability values of less than 0.05 were considered significant.

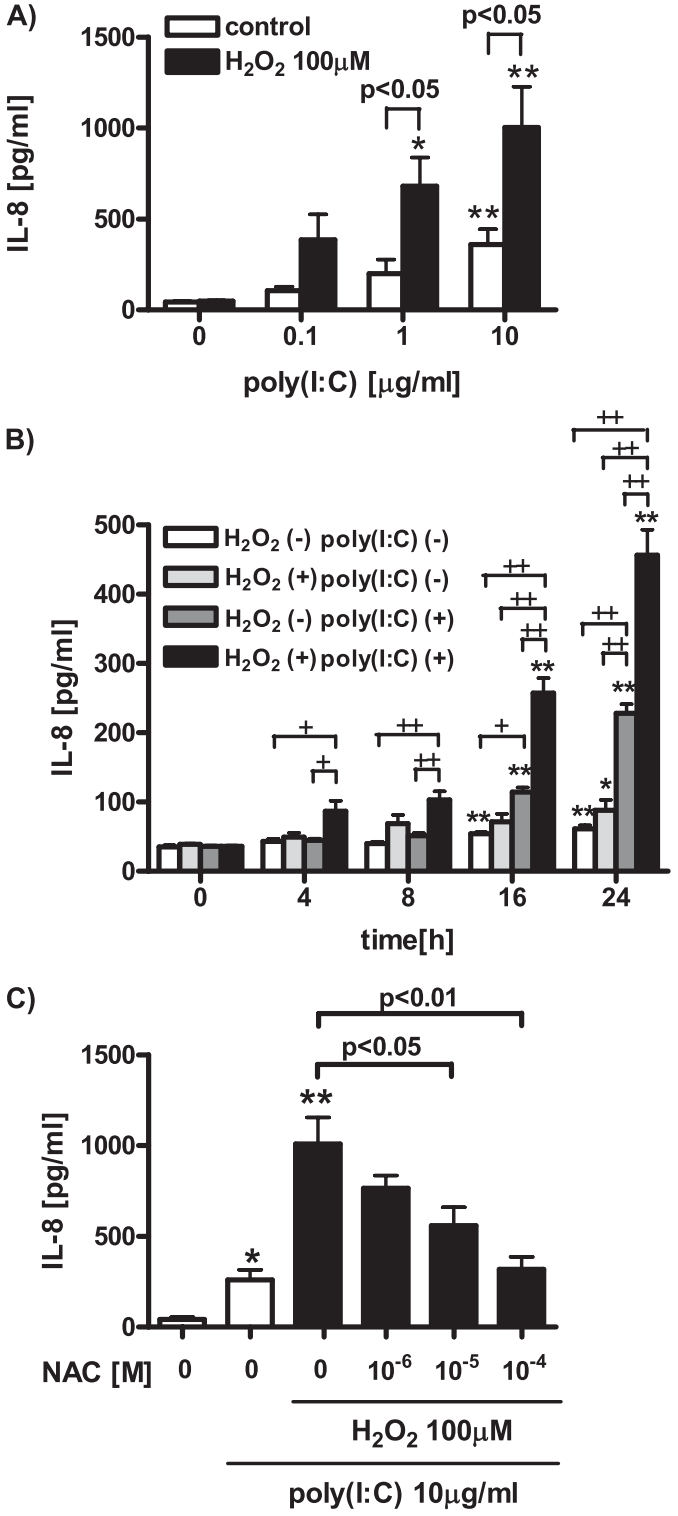

Figure 3. Effect of H_2O_2 on IL-8 release and effect of N-acethylcysteine (NAC) on H_2O_2-augmented IL-8 release in poly(I:C)-treated cells. (*A*) BEAS-2B cells were treated with 100 μM H_2O_2 or vehicle 30 minutes before the treatment with various concentrations of poly(I:C). After 24 hours, supernatants were harvested and assayed for IL-8. (*B*) Cells were treated with 100 μM H_2O_2 or vehicle 30 minutes before the treatment with 10 μg/ml poly(I:C). At various time points after the incubation, supernatants were harvested and assayed for IL-8. (*C*) Various concentrations of NAC were added 2 hours before H_2O_2 treatment, and then cultured in the presence of 10 μg/ml poly(I:C). After 24 hours, supernatants were harvested and assayed for IL-8. The data are expressed as mean values ± SEM for four to six separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with the values of vehicle-treated cells; $^{+}P < 0.05$, $^{++}P < 0.01$ compared with the values of each group.

RESULTS

Expression of TLR3 on Human Bronchial Epithelial Cells and Effect of TLR3 Ligand, Poly(I:C) on IL-8 Release

To confirm whether human bronchial epithelial cells express TLR3, we first examined the expression of TLR3 by immunocytochemistry and immunoblotting. TLR3 was detected in BEAS-2B cells by immunocytochemistry (Figure 1A). TLR3 was detected at approximately 110 kD by immunoblotting in BEAS-2B cells and HBEpC as previously reported (Figure 1B). Next, we investigated the effect of the TLR3 ligand, poly(I:C) on the release of IL-8 from BEAS-2B cells. Poly(I:C) significantly increased the release of IL-8 from the epithelial cells in a dose-dependent manner (Figure 2A). TLR3 has been reported to exist in the endosome and to require an acidic environment for its activation. To confirm whether the effect of poly(I:C) was mediated through TLR, we used bafilomycin, which is an inhibitor of endosomal acidification. Bafilomycin dose-dependently inhibited the poly(I:C)-induced IL-8 release (Figure 2B). We examined the effect of other TLR ligands, including LPS(TLR4), R837(TLR7), and R848(TLR7/8), on the release of IL-8. LPS (Figure 2C), R837 (Figure 2D), or R848 (Figure 2E) had no effect on the release of IL-8 from the epithelial cells.

Effect of H_2O_2 on the Release of IL-8 from BEAS-2B Cells after Poly(I:C) Stimulation

To elucidate the effect of oxidative stress on the IL-8 release, the cells were treated with H_2O_2 in the current study. Because treatment with less than 200 μM H_2O_2 did not affect the cell viability and the release of IL-8 (*see* Figures E1A and E1B in the online supplement), we used H_2O_2 at the concentration of 100 μM or 150 μM in the current study. One hundred micromoles or 150 μM H_2O_2 significantly potentiated the release of IL-8 in the presence of poly(I:C) in a time- and concentration-dependent manner (Figures 3A, 3B, and E1B). The potentiation by H_2O_2 was significantly inhibited by pretreatment with NAC in a concentration-dependent manner (Figure 3C).

Effect of H_2O_2 on the Nuclear Translocation of NF-κB and IRF3 Induced by Poly(I:C)

To explore the mechanism for the H_2O_2-augmented IL-8 release in the poly(I:C)-treated cells, the effect of H_2O_2 on the translocation of NF-κB and IRF-3 into the nucleus was evaluated. Treatment with H_2O_2 caused a small but significant increase in NF-κB p65 translocation into the nucleus (Figures 4A–4D). However, H_2O_2 significantly potentiated NF-κB p65 translocation into the nucleus in the presence of poly(I:C) in a time- and concentration-dependent manner (Figures 4A–4D). The potentiation of NF-κB translocation was not observed after 24 hours of H_2O_2 treatment (Figure E2). H_2O_2 also significantly potentiated NF-κB DNA binding activity in the presence of poly(I:C) (Figure 4E). Furthermore, pretreatment with MG132 significantly inhibited the H_2O_2-augmented IL-8 release in the poly(I:C)-treated cells (Figure 4F).

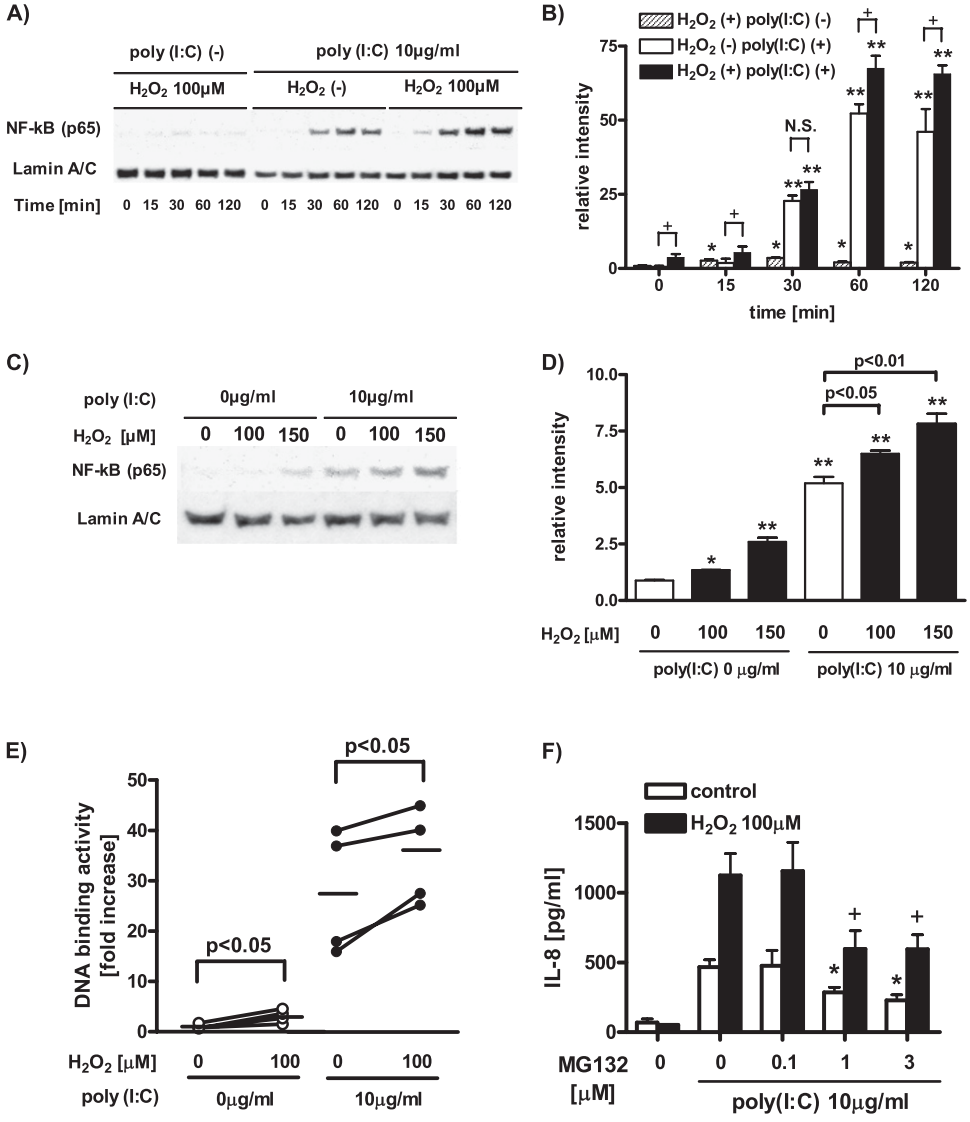

Figure 4. Effect of H_2O_2 on poly(I:C)-induced nuclear factor (NF)-κB translocation into nucleus and DNA binding activity and effect of MG132 on H_2O_2-augmented IL-8 release in poly(I:C)-treated cells. (*A*) Cells were treated with 100 μM H_2O_2 or vehicle 30 minutes before the treatment with 10 μg/ml poly(I:C). At various time points, the nuclear fraction of cell lysates was obtained. NF-κB p65 translocation into the nucleus was evaluated by immunoblotting. (*B*) Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the NF-κB band intensity by the appropriate lamin A/C band intensity. $^*P < 0.05$, $^{**}P < 0.01$ compared with the values of vehicle-treated cells at 0 minutes; $^+P < 0.05$ compared with the values of each group. (*C*, *D*) Dose-dependent effect of H_2O_2 on poly(I:C)-induced NF-κB translocation was assessed by immunoblotting (*C*). The relative intensity of each band was assessed by densitometry (*D*). $^*P < 0.05$, $^{**}P < 0.01$ compared with the values of vehicle-treated cells. (*E*) NF-κB DNA binding activity was evaluated by ELISA. (*F*) Cells were treated with 10 μg/ml poly(I:C) with 100 μM H_2O_2 or vehicle in the presence of MG132. After 24 hours, the supernatants were assayed for IL-8 by ELISA. $^*P < 0.05$, $^+P < 0.05$ compared with the values of vehicle-pretreated poly(I:C)-treated cells. The data are expressed as mean values ± SEM for three to six separate experiments.

Ten micrograms per milliliter of poly(I:C) significantly enhanced IRF-3 translation into the nucleus (Figures 5A and 5B). However, pretreatment with H_2O_2 caused no potentiation of IRF-3 translocation into the nucleus in the poly(I:C)-treated cells (Figures 5A and 5B). Since there is no available inhibitor for IRF-3, siRNA, for IRF3 was used to estimate the inhibitory effect on the release of IL-8. Although treatment with siRNA eliminated the IRF-3 protein (Figure 5C), IRF-3 knockdown did not affect the H_2O_2-augmented IL-8 release in the poly(I:C)-treated cells (Figure 5D).

Effect of H_2O_2 on the Expression of TLR3

To explore another mechanism of the H_2O_2-potentiated IL-8 release in the poly(I:C)-treated cells, the effect of H_2O_2 on the expression of TLR3 in the epithelial cells was evaluated. Treatment with 100 μM H_2O_2 or 10 μg/ml poly(I:C) alone did not affect the expression of TLR3 (Figure 6A). However, combination of poly(I:C) and H_2O_2 significantly increased the TLR3 expression in a time-dependent manner (Figures 6A and 6B).

Effect of Dexamethasone on the Potentiated IL-8 release by H_2O_2 and TLR3 Expression in BEAS-2B Cells

Because systemic steroid has been reported to be effective for exacerbations of COPD (35, 36), we investigated the effect of steroid on the H_2O_2-potentiated IL-8 release in the poly(I:C)-treated cells. A quantity of 10^{-7} to 10^{-6} M DEX significantly inhibited the poly(I:C)-augmented IL-8 release in the presence or absence of H_2O_2 (Figure E3). At 10^{-6} M DEX, there was still a significant difference in the poly(I:C)-augmented IL-8 release between the H_2O_2-treated and vehicle-treated groups (Figure E3). Treatment with DEX did not affect the H_2O_2-potentiated TLR3 expression in the poly(I:C)-treated cells (Figure 6C).

Effect of H_2O_2 and Poly(I:C) on the Release of IL-8, NF-κB Translocation, and TLR3 Expression in HBEpC

We confirmed the potentiation by H_2O_2 on the IL-8 release, NF-κB translocation, and TLR3 expression in HBEpC. Treatment with H_2O_2 slightly but significantly augmented IL-8 release in the poly(I:C)-treated cells (Figure 7A). H_2O_2 also significantly potentiated the poly(I:C)-augmented NF-κB p65 translocation and TLR3 expression in the poly(I:C)-treated cells as well as in BEAS-2B cells (Figures 7B and 7C).

DISCUSSION

In the current study, we demonstrated the expression of TLR3 on BEAS-2B cells and HBEpC and that a synthetic dsRNA,

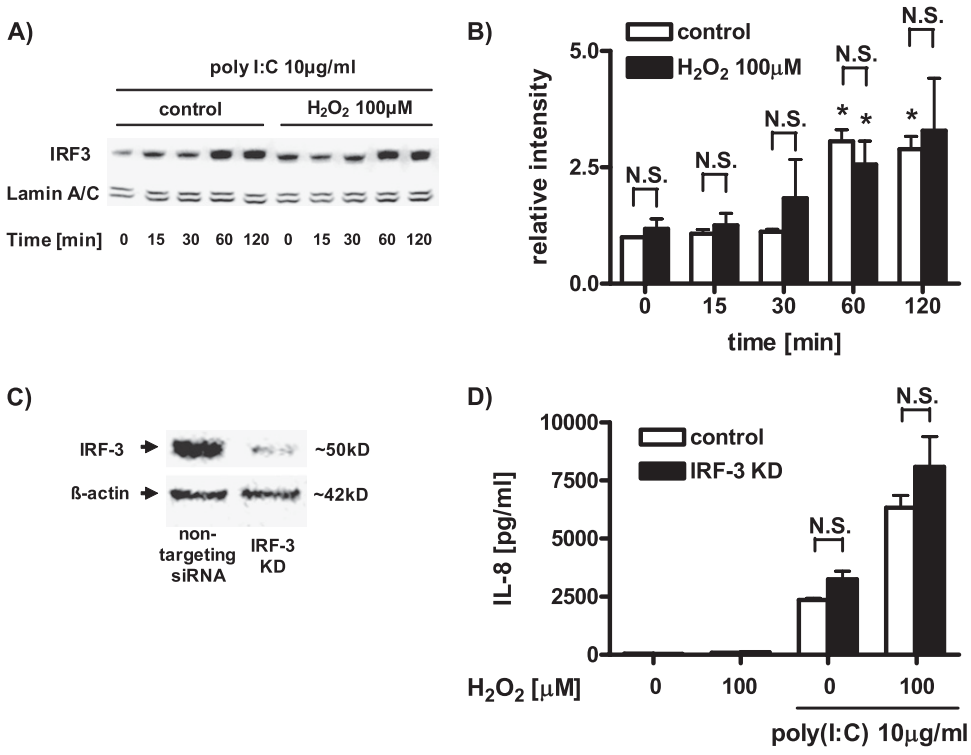

Figure 5. Effect of H_2O_2 on poly(I:C)-induced interferon regulatory factor-3 (IRF-3) translocation and effect of IRF-3 silencing with siRNA on H_2O_2-potentiated IL-8 release in poly(I:C)-treated cells. Cells were treated with 100 μM H_2O_2 or vehicle and then 10 μg/ml poly(I:C) were added. At various time points, the nuclear fraction of cell lysates was obtained. (*A*) IRF-3 translocation into the nucleus was evaluated by immunoblotting. (*B*) Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the IRF-3 band intensity by each appropriate lamin A/C band intensity. $*P < 0.05$; compared with the values of control at 0 minutes. Cells were treated with nontargeting siRNA or IRF-3 siRNA for 6 hours. Cells were further incubated with 10 μg/ml poly(I:C) or vehicle in the presence of 100 μM H_2O_2 for 24 hours. Supernatants were harvested and assayed for IL-8. (*C*) IRF-3 expression was evaluated by immunoblotting. The amount of IRF-3 was assessed by densitometry. (*D*) Effect of IRF-3 silencing with siRNA on H_2O_2-potentiated IL-8 release from poly(I:C)-treated cells. The data are expressed as mean values ± SEM for three to six separate experiments. IRF-3 KD, IRF-3 knock down; N.S., not significant.

poly(I:C), induced IL-8 release from the epithelial cells. Pretreatment with H_2O_2 potentiated the poly(I:C)-augmented IL-8 release, and the antioxidant NAC reversed this potentiation, suggesting that oxidative stress potentiates the dsRNA-induced IL-8 release from human bronchial epithelial cells. We also showed that H_2O_2 potentiated the poly(I:C)-induced NF-κB translocation, not IRF-3, into the nucleus and NF-κB DNA binding activity, and suppression of NF-κB by MG132 inhibited the H_2O_2-potentiated IL-8 release in the poly(I:C)-treated cells. Furthermore, treatment with H_2O_2 plus poly(I:C) increased the TLR3 expression compared with the basal condition. These H_2O_2-mediated potentiations of the TLR3 responses were also confirmed in primary human airway epithelial cells. These data suggest that oxidative stress potentiates the poly(I:C)-augmented IL-8 release in airway epithelial cells through NF-κB activation, and this potentiation might be partly explained by the increased TLR3 expression.

During exacerbations of COPD, levels of IL-8 in sputum and exhaled breath condensate were elevated (28, 30, 31) and neutrophil infiltration was augmented in the airways (32, 33). Although the mechanism for this enhanced neutrophilic inflammation has not been fully elucidated yet, our findings may explain the mechanism. Generally, greater amounts of oxygen radicals are produced during exacerbations compared with the stable condition (12, 13). When viral infections occur, TLR3-mediated IL-8 release might further increase in the airways and consequently lead to excessive neutrophil accumulation.

In the current study, H_2O_2 augmented the poly(I:C)-induced translocation of NF-κB p65 into the nucleus and suppression of NF-κB by MG132 inhibited the H_2O_2-augmented IL-8 release in the poly(I:C)-treated cells. These results suggest that the augmentation of IL-8 release by H_2O_2 is due to the potentiation of poly(I:C)-induced NF-κB activation. In previous studies, H_2O_2 has been reported not only to directly induce the NF-κB transcriptional activity (37), but also enhance NF-κB activation in response to proinflammatory cytokines (38, 39). The molecular mechanisms by which H_2O_2 potentiates NF-κB signaling remain uncertain, but several possible mechanisms have been reported. Takada and coworkers showed that H_2O_2 activates NF-κB through tyrosine phosphorylation of IkBα and serine phosphorylation of the p65 subunit of NF-κB (39). Others have also reported that H_2O_2 activates NF-κB via phosphorylation of serine residues in the IkB kinases (IKKs) (40). Recently, H_2O_2 has been reported to prolong nuclear localization of NF-κB in proinflammatory cytokine-activated cells by suppressing the negative regulation by IkBα and other proteases (41). In our current study, H_2O_2-augmented NF-κB p65 translocation to the nucleus in poly(I:C)-treated cells continued until 120 minutes compared with the control, which might be consistent with the previous study (41). We showed that poly(I:C) induced the translocation of IRF3 into the nucleus, but H_2O_2 did not enhance this translocation. Further silencing of IRF-3 did not affect the potentiation of IL-8 release. Together, potentiation of IL-8 release is mainly mediated via NF-κB but not IRF-3 signaling.

In the present study, we showed that the combination of poly(I:C) and H_2O_2 significantly enhanced TLR3 expression in airway epithelial cells. This result suggests that oxidative stress might affect the regulation of TLR3 expression. Previous reports have shown that poly(I:C) augments TLR3 expression in epithelial cells through a type I IFN-dependent mechanism (21, 42). Oxidative stress also has been reported to enhance TLR3 expression in adult human astrocytes (43). These reports might support the current results. Recently, increased TLR3 expression has been observed in airway epithelial cells from patients with acute respiratory distress syndrome in which

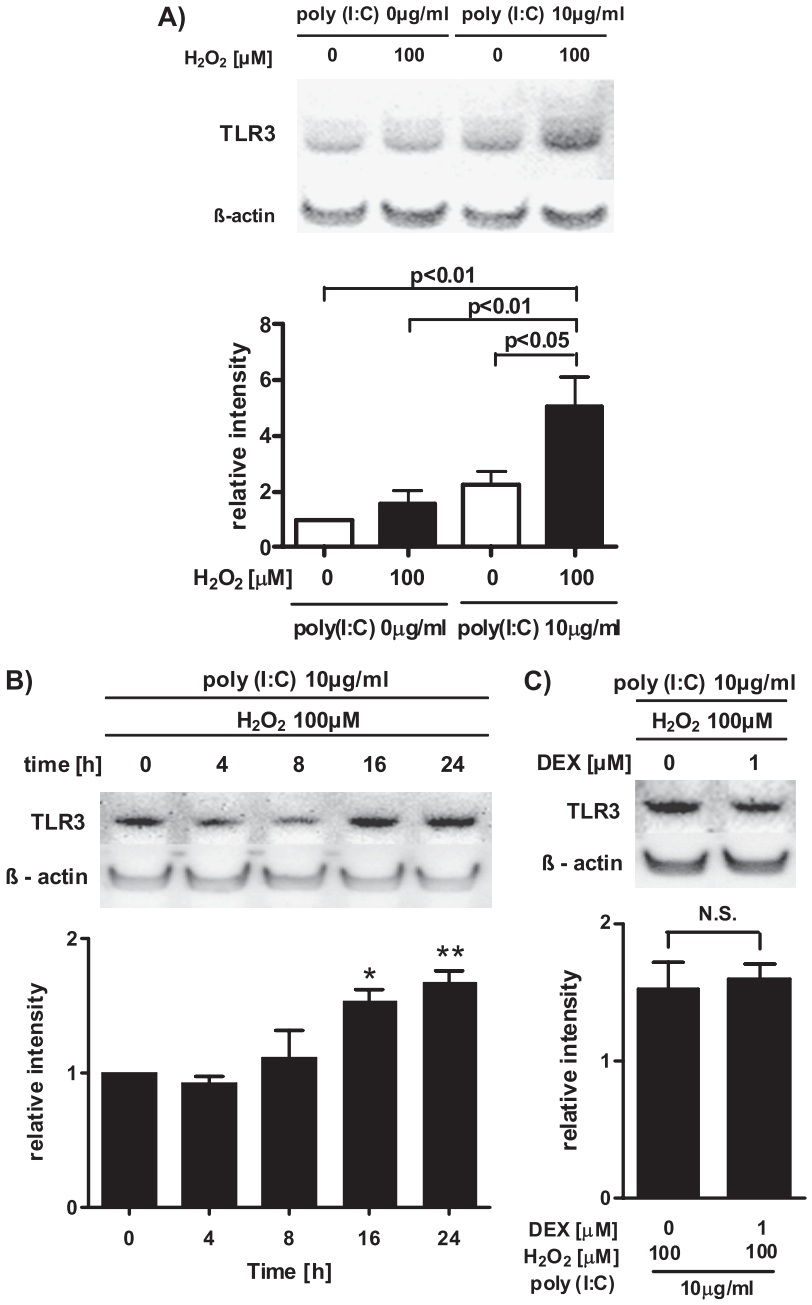

Figure 6. Effect of H_2O_2 on the expression of TLR3 and effect of dexamethasone (DEX) on H_2O_2-augmented TLR3 expression in poly(I:C)-treated cells. (*A*) Cells were treated with 100 μM H_2O_2 or vehicle and then 10 μg/ml poly(I:C) were added. After 24 hours, whole cell lysates were obtained. The expression of TLR3 was evaluated by immunoblotting. Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the TLR3 band intensity by each appropriate β-actin band intensity. (*B*) Cells were treated with 100 μM H_2O_2 and then 10 μg/ml poly(I:C) were added. At various time points, whole cell lysates were obtained and TLR3 expression was evaluated by immunoblotting. (*C*) Cells were treated with 1 μM DEX or vehicle 30 minutes before the treatment with 100 μM H_2O_2 and 10 μg/ml poly(I:C). After 24 hours, whole cell lysates were obtained. The expression of TLR3 was evaluated by immunoblotting. The data are expressed as mean values ± SEM for four to five separate experiments.

airway cells are exposed to a hyperoxic condition (44), suggesting that oxidative stress might modulate TLR3 expression. However, there have not been any studies to clarify the mechanism of this modulation in epithelial cells. In the current study, DEX did not affect the H_2O_2-potentiated TLR3 expression. This might suggest that steroid-sensitive signals such as NF-κB are not involved in the mechanism of H_2O_2-potentiated TLR3 expression. However, this mechanism remains unclear and further studies are needed.

In the current study, we investigated the time course of potentiation by H_2O_2 in IL-8 release and TLR3 expression to examine whether potentiation in the IL-8 release by H_2O_2 is due to up-regulation of TLR3 expression in the poly(I:C)-treated cells. While the TLR3 expression was potentiated by H_2O_2 at 16 hours or later, potentiation of the IL-8 release occurred at 4 to 8 hours or later. These results suggest that the potentiation of the IL-8 release by H_2O_2 was mainly mediated by the potentiation of NF-κB activation. However, at 16 hours or later, the enhanced TLR3 expression might contribute to the potentiation of IL-8 release by H_2O_2.

During acute exacerbations, patients with COPD usually are treated with steroids, and steroids have been reported to improve the severity of exacerbations (35, 36). In this study, DEX dose-dependently inhibited the poly(I:C)-induced IL-8 release from epithelial cells. The inhibition of the IL-8 release by DEX in the H_2O_2-pretreated and poly(I:C)-exposed cells was less than in the vehicle-pretreated and poly(I:C)-exposed cells, suggesting that oxidative stress reduces the effect of steroid on the IL-8 release. The acquirement of steroid resistance under oxidative stress has been reported to occur in macrophages and epithelial cells via inactivation of histone deacetylase 2 (HDAC2) (34, 45). This inactivation of HDAC2

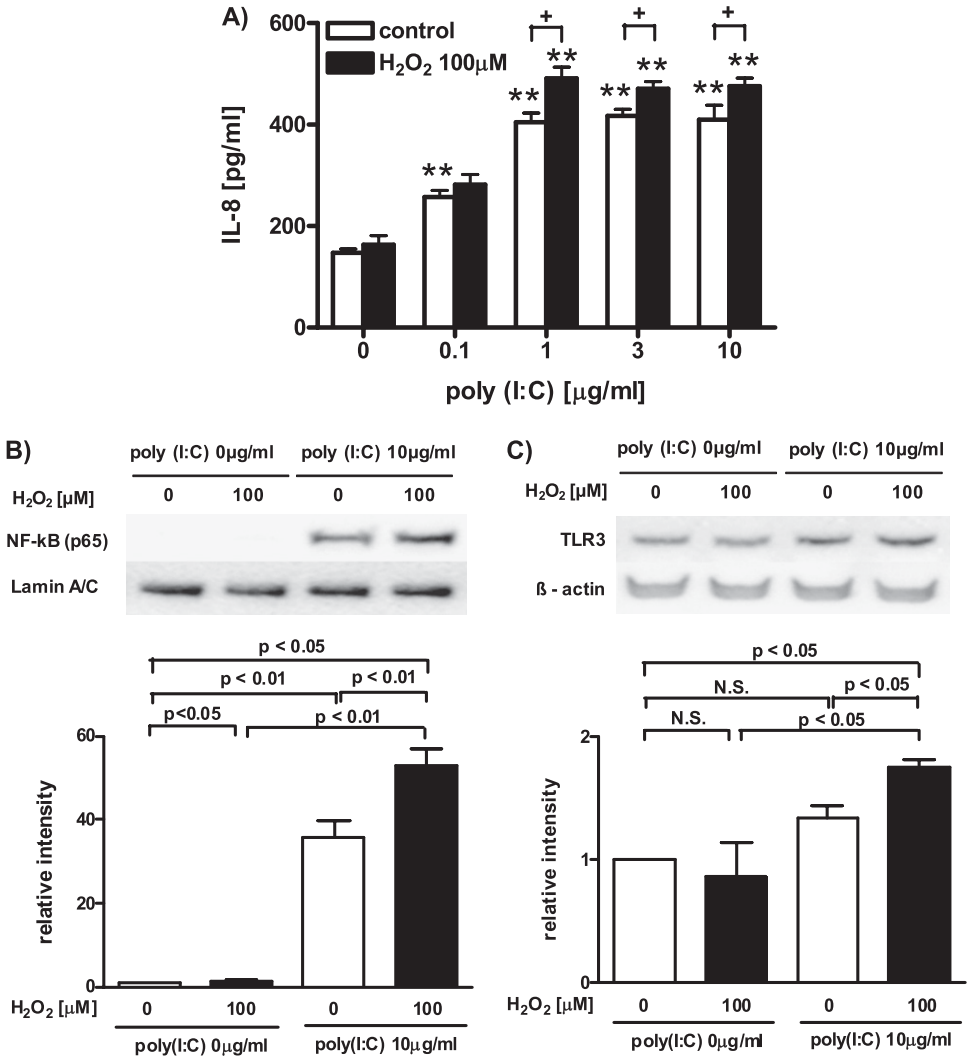

Figure 7. Effect of H_2O_2 on IL-8 release, poly(I:C)-induced NF-κB translocation and TLR3 expression in poly(I:C)-treated primary human bronchial epithelial cells (HBEpC). Four different strains of primary cells were treated with 100 μM H_2O_2 or vehicle 30 minutes before the treatment with 10 μg/ml poly(I:C). (*A*) After 24 hours, supernatants were harvested and assayed for IL-8. The data are expressed as mean values ± SEM for four separate experiments. (*B*) After 60 minutes, the nuclear fraction of cell lysates was obtained. NF-κB p65 translocation into the nucleus was evaluated by immunoblotting. (*C*) After 24 hours, whole cell lysates were obtained. Expression of TLR3 was evaluated by immunoblotting. The data are expressed as mean values ± SEM for four separate experiments. $^{**}P < 0.01$ compared with the values of vehicle-treated cells; $^{+}P < 0.05$ compared with the values of each group.

by oxidative stress might explain our result that DEX was less effective in the H_2O_2-treated cells.

Recently, several reports have shown the role of TLR activation under oxidative stress. Chen and colleagues have reported that the production of cytokines and chemokines such as TNF-α, IL-6, and IL-8 is increased in alveolar macrophages from smokers after poly(I:C) stimulation compared with those from nonsmokers (46). In a murine model, depletion of TLR3 was demonstrated to inhibit the smoking-enhanced airway inflammation and remodeling after influenza virus infection (47). This result suggests that oxidative stress from cigarette smoke might enhance the TLR3 signaling, which is consistent with our results.

In a recent report, Zmijewski and coworkers have shown that H_2O_2 has an anti-inflammatory effect and prevents the activation of NF-κB (48), which is inconsistent with the result of our current study. They demonstrated that a catalase inhibitor, aminotriazole, increased the production of reactive oxygen species in murine neutrophils and attenuated LPS-induced acute lung injury in a murine model. This effect was due to the alleviation of NF-κB activity and proinflammatory cytokine production in neutrophils. The discrepancy from our current results might be explained as follows. First, we administered H_2O_2 exogenously to the airway epithelial cells, whereas they evaluated the role of endogenously produced H_2O_2 by the inhibition of catalase in the reactive oxygen species production from neutrophils. Moreover, the inhibition of catalase might affect the antioxidant system, including glutathione and superoxide dismutase. Second, they evaluated the role of catalase inhibition in NF-κB signaling and proinflammatory cytokine production in murine neutrophils. We assessed the role of H_2O_2 in NF-κB signaling and IL-8 production in human bronchial epithelial cells. Differences in the species and cell type might have affected the results. Third, we assessed TLR3 signaling in the current study. They showed the effect of catalase inhibition on lung inflammation induced by LPS treatment. In general, LPS stimulates TLR4; therefore, the discrepancies in the results might be due to the difference in activated TLRs.

In the current study, TLR4, TLR7, and TLR7/8 ligands did not enhance IL-8 release from BEAS-2B cells. Previously, Sha and colleagues have shown that 1 μg/ml LPS treatment slightly potentiated the IL-8 release from the cells (49). Generally, this concentration appears quite high. In the current study, we used 100 ng/ml LPS to stimulate IL-8 release in BEAS-2B cells. Therefore, the lack of augmentation in IL-8 release by LPS could be due to the difference in the LPS concentration. In a recent report, Koff and coworkers have shown that TLR1/2, TLR5, and TLR6/2 ligands augment IL-8 release, albeit to a lesser extent than TLR3 stimulation in human bronchial

epithelial cells (50). Therefore, not only TLR3 ligand but also other TLR ligands could stimulate IL-8 release in human bronchial epithelial cells. The aim of this study was to clarify the role of TLR3 activation induced by viral infection under oxidative stress. Therefore, we investigated only TLR3 signaling, not other TLRs.

There are several limitations in the current study. First, H_2O_2 potentiated IL-8 production in the poly(I:C)-treated cells through the activation of NF-κB. However, it remains unclear whether the potentiation of H_2O_2 in NF-κB activation is synergetic or not. Second, TLR3 was up-regulated by the H_2O_2 plus poly(I:C) treatment for 24 hours in epithelial cells in the current study. However, the role of the up-regulated TLR3 in the IL-8 release could not be fully elucidated. Because TLR3 expression was augmented at 16 hours or later by treatment with H_2O_2 and poly(I:C), the augmentation of the expression may affect the IL-8 release.

In conclusion, we showed that oxidative stress potentiates the poly(I:C)-induced IL-8 release from airway epithelial cells through the augmentation of NF-κB signaling and that this potentiation might be partly explained by the enhancement of TLR3 expression. These results suggest that oxidative stress augments the neutrophilic inflammation in the airways of patients with COPD during viral-induced exacerbations. Modulation of this pathway may be a therapeutic target for exacerbations of COPD.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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