Figure 4 *(see previous page)*
Effect of H_2O_2 on the R848-induced nuclear factor-kappa B (NF-kB) activation. Cells were treated with or without 50 μM H_2O_2, and then further treated with various concentrations of R848 for 60 min. Phosphorylated NF-kB p65 was assayed by a flow cytometer (A), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα) protein levels were assayed by western blotting (B). Each band intensity was assessed by densitometry. Relative intensity was calculated as the ratio of the specific band intensity to that of each appropriate β-actin band intensity. (C) PMNs were treated with 1 μM R848 with or without 50 μM H_2O_2 in the presence or absence of MG-132, a proteosome inhibitor. After 24 hrs, the media were assayed for IL-8 by ELISA. All values were mean values ± SEM of three to five separate experiments, and analyzed by ANOVA followed by Bonferroni's test. **p < 0.01, compared with the values of control; ++p < 0.01, compared with the values of H_2O_2-pretreated and vehicle-treated group; NF-kB p65 = nuclear factor-kappa B p65; IkBα = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

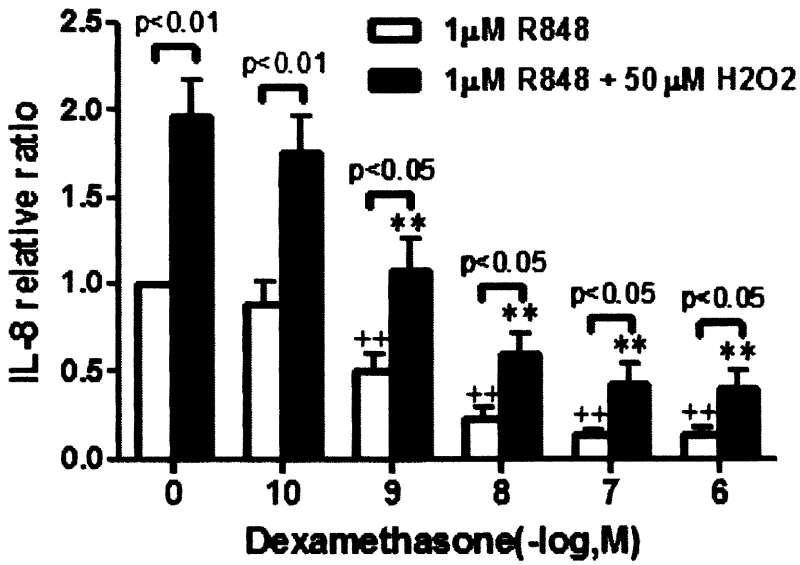

Figure 5
Effect of dexamethasone on the H_2O_2-potentiated IL-8 release in the R848-treated PMNs. PMNs were treated with or without dexamethasone for 30 min before treatment with or without 50 μM H_2O_2. Then, cells were treated with R848 for 24 hours. Media were assayed for IL-8 by ELISA. Vertical axis: IL-8 relative release (-fold increase). IL-8 relative ratio was calculated as follows: IL-8 relative ratio = IL-8 levels in the various conditions/IL-8 levels in the vehicle-pretreated and R848-treated condition. All values are mean values ± SEM of six separate experiments. ++p < 0.01, compared with the values of vehicle pretreated R848 treated group; **p < 0.01, compared to the values of H_2O_2 pretreated R848 treated group.

as the ligand [16,21,22]. The stimulation of TLR 7/8 by R848 might be different from that of single strand RNA virus infection. In the current study, we attempted to elucidate the effects of oxidants on the TLR8 signaling. To accomplish this, we used R848 for the following reasons. First, R848 is a stable agent and is easy to handle compared with single strand RNA. Second, R848 does not have any other effect except TLR 7/8 stimulation. Indeed, the R848 signaling was abolished by treatment with bafilomycin, an inhibitor of endosomal acidification. Therefore, the findings in the current study seemed to be mediated by TLR8 signaling.

In conclusion, we have shown that the TLR8-mediated neutrophilic responses in healthy never-smoking subjects were markedly potentiated by oxidative stress, and this potentiation was mediated by enhanced NF-kB activation. These results suggested that oxidative stress might potentiate the neutrophilic inflammation during viral infection.

Abbreviations

COPD: Chronic obstructive pulmonary disease; TLR8: Toll-like receptor 8; H_2O_2: Hydrogen peroxide; NF-kB p65: Nuclear factor-kappa B p65; IkBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MyD88: Myeloid differentiation primary response gene 88; TRAF6: Tumor necrosis factor receptor-associated factor 6.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SY carried out the data analysis and drafted the manuscript. AK, HS, and MI participated in the design of the original study, and contributed substantially to the manuscript. TI, MK, RT, KA, TH, KM and YM assisted with data analysis and interpretation, and supervised statistical analysis.

Additional material

Additional file 1

*Effect of MG-132 on the R848-induced nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα) degradation. PMNs were incubated with or without 10 μM MG-132, a proteosome inhibitor, and then further treated with various concentrations of R848 for 60 min. The cytoplasmic fraction of cell lysates were used for estimating the protein levels of IKBα by western blotting. Each band intensity was assessed by densitometry. Relative intensity was calculated as the ratio of specific band intensity to that of each appropriate β-actin band intensity. All values are mean values ± SEM of three separate experiments. **$p < 0.01$; compared with the values of vehicle-treated group, IkBα = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, n.s. = not significant.*

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N-acetyl-L-cysteine inhibits TGF-β_1-induced profibrotic responses in fibroblasts

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ABSTRACT

Background: Excessive production of TGF-β_1 plays a key role in the tissue remodeling or fibrotic process observed in bronchial asthma, chronic pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF). TGF-β_1 has been reported to decrease the intracellular glutathione level and stimulate the production of reactive oxygen species.

Objectives: The aim of this study was to evaluate whether the antioxidant N-acetyl-L-cysteine (NAC) can affect TGF-β_1-mediated tissue remodeling in fibroblasts or modulate the production of fibronectin and vascular endothelial growth factor (VEGF) which are believed to be important mediators of tissue repair and remodeling.

Methods: To accomplish this, human fetal lung fibroblasts (HFL-1) were used to assess the effect of NAC on the TGF-β_1-mediated contraction of floating gels and the TGF-β_1-induced mediator production. In addition, the effect of NAC on the TGF-β_1-induced differentiation to myofibroblasts was evaluated by assessing α-smooth muscle actin (α-SMA) expression.

Results: NAC significantly abolished the TGF-β_1-augmented gel contraction (at 3 mM, gel size $63.4 \pm 2.6\%$ vs. $39.1 \pm 4.1\%$; $p < 0.01$) compared with control in a concentration-dependent manner. NAC also significantly inhibited the TGF-β_1-augmented fibronectin ($p < 0.01$) and VEGF ($p < 0.01$) production in the media of both the three-dimensional gel and monolayer culture. Furthermore, NAC reversed the TGF-β_1-stimulated α-SMA expression ($p < 0.01$).

Conclusion: These results suggest that NAC can affect the TGF-β_1-induced tissue remodeling or fibrotic process in vitro.

1. Introduction

Fibroblasts play a pivotal role in the normal wound healing or tissue repair process [1], whereas excessive stimuli for fibroblasts lead to the tissue remodeling or fibrous tissue that is observed in bronchial asthma [2], chronic obstructive pulmonary disease (COPD) [3] and idiopathic lung fibrosis (IPF) [4].

Transforming growth factor (TGF)-β_1 is a multifunctional cytokine in a variety of physiological and pathological processes, including fibroblast repair responses [5,6]. TGF-β_1 regulates the fibroblast migration, proliferation, differentiation and production of matrix. In addition, TGF-β_1 stimulates the fibroblast-mediated contraction of extracellular matrix [7].

N-acetylcysteine (NAC) is a non-toxic aminothiol that has a strong reductive capacity [8]. Because NAC is not only a precursor of GSH but also shows a direct ROS-scavenging capacity, NAC has been widely used as a potent antioxidant. In cultured fibroblasts, NAC inhibited the TGF-β_1-stimulated collagen production [9]. Moreover, aerosolized administration of NAC attenuates the lung fibrosis induced by bleomycin in mice [10]. These results suggest that antioxidant may suppress TGF-β_1 signaling in vitro and in vivo.

Recently, Meurer et al. clearly showed that NAC itself reduced the disulfide bonds of TGF-β_1 and changed the bioactive form (dimmer) to the inactive form (monomer) [11]. Moreover, NAC changed the binding activity of TGF-β_1 to its receptor in hepatic stellate cells [11], suggesting that the effect of antioxidant NAC is based on a direct blockade of TGF-β_1 function and signaling. However, it has not been fully elucidated whether NAC can modulate the TGF-β_1-induced tissue repair, mediator production and differentiation in human lung fibroblasts.

In the current study, we used a fibroblast-mediated collagen gel contraction assay as a tissue repair model in the current study and assessed the effect of NAC on the TGF-β_1-stimulated collagen gel contraction. We investigated whether NAC affected the production of

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fibronectin and vascular endothelial growth factor (VEGF), which are believed to be important mediators of repair and remodeling. Furthermore, we evaluated the effect of NAC on the TGF-β-induced differentiation to myofibroblasts by assessing α-smooth muscle actin (α-SMA) expression.

2. Materials and methods

2.1. Materials

Native type I collagen [rat tail tendon collagen (RTTC)] was extracted from rat tail tendons by a previously published method [12]. Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. Repeated washing with Tris-buffered saline (0.9% NaCl and 10 mM Tris, pH 7.5) was followed by dehydration and sterilization with 50%, 75%, 95% and pure ethanol. Type I collagen was then extracted in 6 mM hydrochloric acid at 4 °C. The collagen concentration was determined by weighing a lyophilized aliquot from each lot of collagen solution. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consistently demonstrated no detectable proteins other than type I collagen.

Commercially available reagents were obtained as follows: TGF-β_1 was from R&D Systems (Minneapolis, MN); N-acetylcysteine (NAC), 3,3′,5,5′-tetramethyl benzidine (TMB), monoclonal anti-human fibronectin antibody, and polyclonal anti-human fibronectin antibody were purchased from Sigma (St. Louis, MO); Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were from Invitrogen Life Technologies (Grand Island, NY).

2.2. Cell culture

Human fetal lung fibroblast (HFL-1) cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured on tissue culture dishes (Falcon; Becton–Dickinson Labware, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 mg/ml penicillin, 250 μg/ml streptomycin and 2.5 μg/ml fungizone. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and passaged every 4–5 days at a 1:4 ratio. HFL-1 cells were used between the 14th and 18th passages. To evaluate mediator production in a monolayer culture, the cells were seeded in 6-well tissue culture plates at a cell density of 1×10^5/ml. At 90% confluence, the cells were treated with various concentrations of NAC in serum-free DMEM (SF-DMEM) in the presence or absence of TGF-β_1. The supernatants of the monolayer culture were harvested on day 3 and stored at −80 °C until later assay.

2.3. Western blotting

Immunoblotting of α-SMA was performed. To detect α-SMA, the cells were seeded in 60 mm dishes at a density of 1×10^5 cell/ml and cultured for 24 h, and then the medium was replaced with SF-DMEM for 24 h. The cells were pretreated with or without NAC for 2 h and then further treated with vehicle or TGF-β_1 for 48 h. The cells were then harvested and homogenized in cell lysis buffer. All samples were eluted at 97 °C for 5 min in sodium dodecylsulfate (SDS)-PAGE sample buffer. Five milligrams of whole cell lysate for α-SMA was separated by electrophoresis on 12% SDS polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with mouse monoclonal anti-α-SMA antibody (1:5000 dilution; Sigma, St. Lois, MO) at 4 °C overnight. Bound antibody was visualized using peroxidase-conjugate anti-mouse IgG antibody and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) with Typhoon Scanner (Amersham Biosciences). Densitometry was used to quantify the expression of α-SMA by using NIH image.

2.4. Collagen gel contraction assay

Collagen gels were prepared as described previously [13]. Briefly, RTTC, distilled water and 4× concentrated DMEM were combined so that the final mixture resulted in 0.75 mg/ml collagen, with a physiological ionic strength of 1× DMEM and a pH 7.4. The cells were trypsinized (trypsin–EDTA; 0.05% trypsin, 0.53 mM EDTA-4Na, GIBCO) and suspended in SF-DMEM. The cells were then mixed with the neutralized collagen solution so that the final cell density in the collagen solution was 3×10^5 cells/ml, and the final concentration of collagen was 0.75 mg/ml. Aliquots (0.5 ml/well) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon) and allowed to gel. After gelation was completed, normally within 20 min at room temperature, the gels were gently released from the 24-well tissue culture plates and transferred into 60 mm tissue culture dishes (three gels in each dish), which contained 5 ml of freshly prepared 0.2% FCS containing DMEM with or without various concentrations of NAC in the presence or absence of TGF-β_1. The gels were then incubated at 37 °C in a 5% CO_2 atmosphere for 3 days. Gel size was measured using an Optomax V image analyzer (Optomax, Burlington, MA) daily. Data were expressed as percentages of the original gel size.

2.5. Measurement of fibronectin and VEGF by ELISA

Soluble form of fibronectin and VEGF in the media of the collagen gel culture or monolayer culture was determined by ELISA. Quantification of fibronectin was performed as previously described [14]. Briefly, plates were coated with monoclonal anti-fibronectin antibody (1:10000 dilution) at 4 °C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. After being washed, bound antigen was detected after adding polyclonal anti-human fibronectin antibody (1:2500 dilution) at room temperature for 1 h. After being washed, HRP conjugated anti-rabbit IgG antibody (1:2500 dilution; Rockland, Gilbertsville, PA) was added at room temperature for 1 h. After being washed, bound HRP was detected with 0.1 mg/ml OPD. The reaction was stopped with 8 M H_2SO_4, and the product was quantified at 490 nm with a microreader. Quantification of VEGF was performed as follows. Plates were coated with 50 ng/ml monoclonal anti-human VEGF antibody (R&D system, Minneapolis, MN) at 4 °C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. After being washed, bound antigen was detected after adding 100 ng/ml biotinylated-anti-human VEGF antibody (R&D system, Minneapolis, MN) for 1 h at room temperature. After being washed, HRP–streptavidin (1:20000 dilution; Zymed Laboratories, San Francisco, CA) was then added for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H_2SO_4, and the product was quantified at 450 nm with a microreader.

2.6. Statistical analysis

Data were expressed as means ± SEM. Experiments with multiple comparisons were evaluated by one way analysis of variance (ANOVA) followed by Bonferroni's test to adjust for multiple comparisons. Probability values of less than 0.05 were considered significant.

3. Results

3.1. Effect of NAC on the TGF-β_1-augmented collagen gel contraction mediated by fibroblasts

To evaluate whether NAC can affect the TGF-β_1-induced tissue repair process, we assessed the effect of NAC by using a collagen gel contraction assay. The cells were pretreated with various

concentrations of NAC for 2 h prior to treatment with TGF-β_1. TGF-β_1 significantly augmented the three-dimensional (3D) collagen gel contraction compared with control on day 3 (at 100 pM, gel size was 39.1 ± 4.1% vs. 61.9 ± 1.1% of initial size; $p < 0.01$, Fig. 1A, B). NAC (0.3–3 mM) significantly blocked the TGF-β_1-augmented collagen gel contraction on day 3 in a concentration-dependent manner (at 3 mM, gel size was 63.4 ± 2.6% vs. 39.1 ± 4.1%; $p < 0.01$, Fig. 1A, B).

3.2. Effect of NAC on fibronectin and VEGF release by fibroblasts in the media of collagen gels and monolayer culture

To evaluate the effect of NAC on fibronectin production, the fibronectin level in the media of the collagen gel and monolayer cultures was measured by ELISA. TGF-β_1 significantly increased fibronectin release in both the 3D gel ($p < 0.01$, Fig. 2A, left panel) and monolayer culture ($p < 0.01$, Fig. 2A, right panel). NAC significantly blocked TGB-β_1-augmented fibronectin release in a concentration-dependent manner in both the 3D gel ($p < 0.01$, Fig. 2A, left panel) and monolayer culture ($p < 0.01$, Fig. 2A, right panel).

We also assessed the effect of NAC on VEGF production by fibroblasts. As shown in Fig. 2B, TGF-β_1 significantly increased VEGF release in both the 3D gel ($p < 0.01$, Fig. 2B, left panel) and monolayer culture ($p < 0.01$, Fig. 2B, right panel), whereas NAC significantly blocked the TGF-β_1-augmented VEGF release in both the 3D gel ($p < 0.01$, Fig. 2B, left panel) and monolayer culture ($p < 0.01$, Fig. 2B, right panel).

3.3. Effect of NAC on the TGF-β_1-augmented expression of α-SMA

To evaluate whether NAC affects the differentiation to myofibroblasts, we investigated the effect of NAC on the expression of α-SMA, which is a marker of differentiation to myofibroblasts. TGF-β_1 significantly stimulated α-SMA expression in the fibroblasts ($p < 0.01$, Fig. 3A, B). Ten mM NAC abolished the TGF-β_1-stimulated expression of α-SMA ($p < 0.01$, Fig. 3A, B), suggesting that NAC could inhibit the TGF-β_1-stimulated differentiation to myofibroblasts.

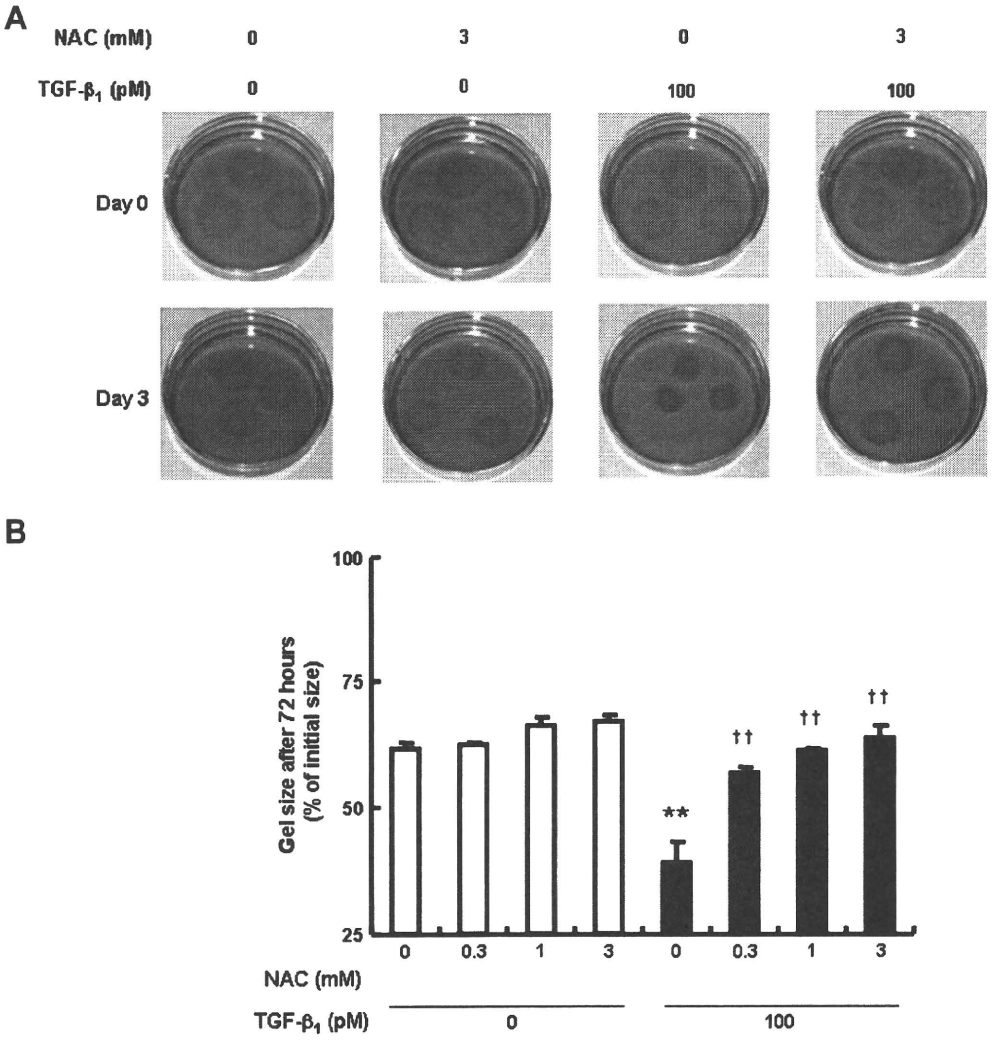

Fig. 1. Effect of NAC on collagen gel contraction mediated by human fibroblasts. Fibroblasts were cast into three-dimensional collagen gels and floated in medium containing various concentrations of NAC for 2 h prior to treatment with TGF-β_1. Gel size was measured daily. Representative photographs of collagen gel contraction assay are presented (Panel A). Gel size after 72 h is presented (Panel B). Panel B: vertical axis: gel size after 72 h incubation (% of initial size); horizontal axis: NAC concentration (mM) and TGF-β_1 concentration (pM). All values are mean ± SEM for three separate experiments, each performed in triplicate. $^{**}p < 0.01$; compared with the values of control. $++p < 0.01$; compared with the values of TGF-β_1 treated group.

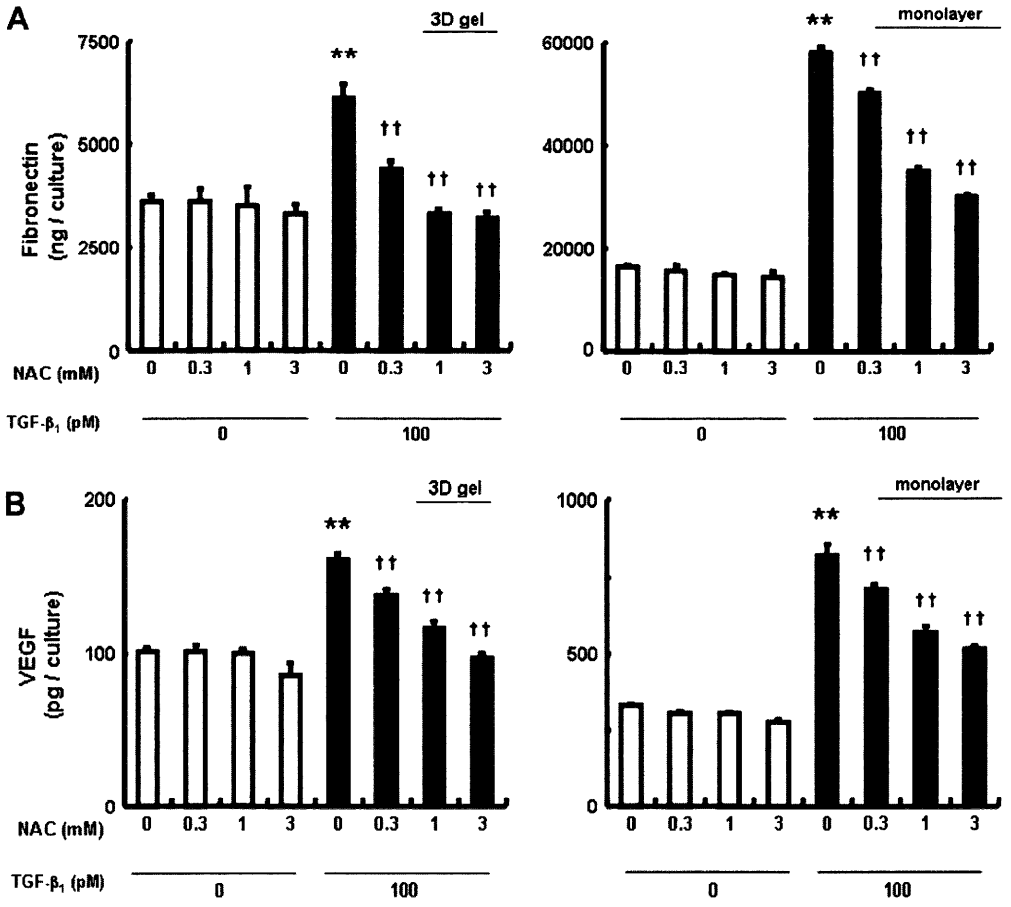

Fig. 2. Effect of NAC on the TGF-β_1-augmented fibronectin release (Panel A) and vascular endothelial growth factor (VEGF) (Panel B) in the culture media. Fibroblasts were cast into collagen gels and maintained in floating culture in medium in the presence of varying concentrations of NAC. After 2 h, gels were treated with or without TGF-β_1 (left panels). In a separate experiment, fibroblasts were cultured until 90% confluence, after which the medium was changed to serum-free DMEM with varying concentrations of NAC. After treatment with or without NAC, the cells were further treated with or without TGF-β_1 (right panels). After 3 days, media were harvested and assayed for fibronectin (A) or VEGF (B) by ELISA. Vertical axes: fibronectin (ng/culture) or VEGF production (pg/culture); horizontal axes: NAC concentration (mM) and TGF-β_1 concentration (pM). All values are mean ± SEM for five separate experiments, each performed in duplicate. $^{**}p < 0.01$; compared with the values of control. $++p < 0.01$; compared with the values of TGF-β_1 treated group.

4. Discussion

The present study demonstrated that NAC inhibited the TGF-β_1-augmented collagen gel contraction, mediator production and α-SMA expression. These results suggest that NAC can affect the tissue repair process in vitro.

NAC is a non-toxic aminothiol, a potent antioxidant, and a synthetic precursor of GSH. NAC is frequently used as an antioxidant in in vitro and in vivo studies. A previous report showed that NAC reduced the intracellular GSH level and production of reactive oxygen species (ROS) [15]. NAC was also reported to inhibit TGF-β_1-stimulated collagen production [9]. These results suggested that intracellular ROS produced by TGF-β_1 was related to TGF-β_1-induced phenomena. Recently, Meurer et al. showed that NAC not only changed TGF-β_1 to the biologically inactive form but also inhibited the binding of TGF-β_1 to its receptor [11]. On the basis of this study, we investigated whether NAC can inhibit TGF-β_1-augmented collagen gel contraction, which is a model of wound healing and tissue remodeling. As shown in Fig. 1, NAC significantly inhibited the TGF-β_1-augmented collagen gel contraction. We also investigated the TGF-β_1-augmented production of fibronectin and VEGF because these mediators were reported to have important roles in wound healing and tissue remodeling [16,17]. NAC inhibited the TGF-β_1-augmented mediator release in the media of both the collagen gel and monolayer culture. Furthermore, we investigated the effect of NAC on the differentiation to myofibroblasts. High concentrations of NAC completely inhibited the TGF-β_1-stimulated α-SMA expression. These data suggested that NAC inhibited not only TGF-β_1 signaling [11] but also the TGF-β_1-mediated wound healing process and differentiation to myofibroblasts.

In the current paper, we confirmed the inhibitory effect of NAC on TGF-β_1-mediated physiological phenomena in vitro. TGF-β_1 signal transduction was completely blocked by NAC because NAC or DTT reduced TGF-β_1 and inhibited the binding to its receptor as a previous paper showed [11]. An other previous study showed that the dimeric form of TGF-β_1 disappeared after incubation with thiols by using mass spectroscopy [18]. Therefore, we need careful evaluation of the effects of reducing agents such as NAC or glutathione.

Fibronectin, a multifunctional glycoprotein involved in tissue remodeling, is a chemoattractant for lung fibroblasts [19] and can be released in increased amounts by fibroblasts [20] and epithelial cells [21] in response to a variety of cytokines. TGF-β_1 is the strongest stimulator of fibronectin release from fibroblasts. Because the excessive production of extracellular matrix is related to tissue remodeling, it appears that NAC can inhibit the TGF-β_1-induced fibronectin production to block abnormal tissue remodeling.

VEGF is a multifunctional growth factor. It was initially characterized as a factor that increases endothelial permeability and induces endothelial cell growth. It is not only essential for the

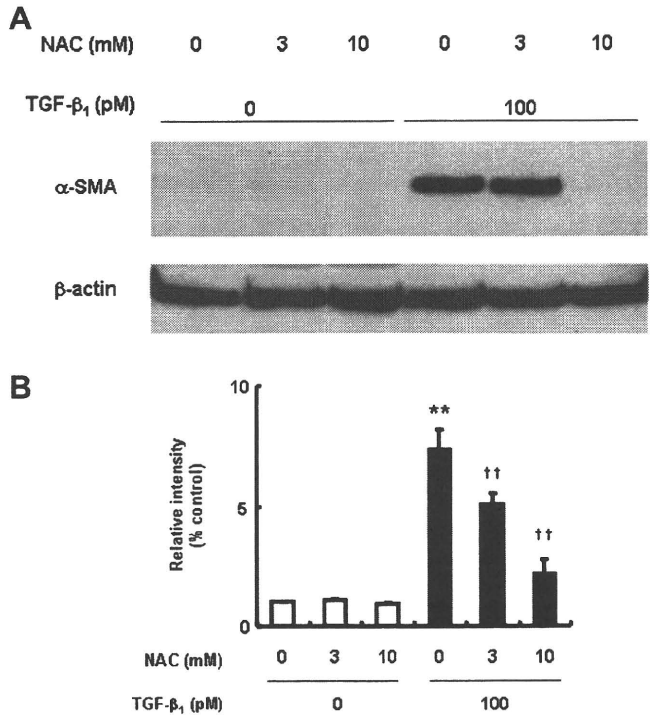

Fig. 3. Effect of NAC on the TGF-β_1-stimulated α-SMA expression. Fibroblasts were treated with or without various concentrations of NAC for 2 h prior to treatment with or without TGF-β_1. After 48 h, the cells were harvested and whole cell lysates were separated by SDS-PAGE. Expression of α-SMA was evaluated by western blotting (A). Intensity of bands detected by western blotting was quantified using NIH image (B). All values are mean ± SEM for three separate experiments. $^{**}p < 0.01$; compared with the values of control. $^{++}p < 0.01$; compared with the values of TGF-β_1 treated group.

normal development of blood vessels in the embryo, but it is also required for the survival of endothelial cells as well as for angiogenesis [22]. We have recently reported that TGF-β_1-enhanced VEGF production via smad3 which is phosphorylated by the activated TGF-β_1 receptor [23]. In the present study, NAC abolished the TGF-β_1-enhanced VEGF production because NAC completely blocked smad3 signal transduction (data not shown). Therefore, NAC may inhibit the TGF-β_1-induced excessive production of VEGF in pathological conditions.

NAC has been used as a potent antioxidant in in vivo studies. In an animal model, NAC could suppress bleomycin-induced lung fibrosis [10]. In a previous paper, high dose administration of NAC with low dose steroids to patients with IPF significantly improved lung function [24]. Although the pathogenesis of IPF is not well understood, TGF-β_1 is a key molecule in the progression of IPF. Based on the current study, NAC may have an effect on lung fibrosis not only as an antioxidant but also a reducing agent for TGF-β_1 itself.

In summary, our data demonstrated that NAC inhibited the TGF-β_1-augmented collagen gel contraction, mediator production, and α-SMA expression in fibroblasts. Since NAC was reported to inactivate TGF-β_1 itself and inhibit the binding to its receptor, its effect on TGF-β_1-induced phenomena in vitro should be carefully evaluated. The effects of NAC on the TGF-β_1-mediated profibrotic change in vivo may be mediated through not only an antioxidant but also an inactivator of TGF-β_1.

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3-Nitrotyrosine inhibits fibroblast-mediated collagen gel contraction and chemotaxis

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ABSTRACT: Reactive nitrogen species induce tissue inflammation and nitrate tyrosine residues of various kinds of proteins. Recent studies have established that the free amino acid form of 3-nitrotyrosine induces cytotoxity and growth inhibition and alters the cellular function in cultured cells. The aim of this study was to evaluate whether 3-nitrotyrosine could affect tissue remodelling in fibroblasts. To accomplish this, human fetal lung fibroblasts (HFL-1) were used to assess the fibroblast-mediated contraction of floating gels and chemotaxis towards fibronectin. In addition, the ability of fibroblasts to release fibronectin, transforming growth factor (TGF)-β1, fibronectin and vascular endothelial growth factor (VEGF) was assessed. 3-Nitrotyrosine significantly inhibited gel contraction ($p<0.01$) compared with control and this inhibition was abolished by nitric oxide synthase (NOS) inhibitor. 3-Nitrotyrosine did not affect TGF-β1 and VEGF but significantly decreased fibronectin release ($p<0.01$) into the media. 3-Nitrotyrosine significantly inhibited chemotaxis towards fibronectin through suppression of $\alpha_5\beta_1$ integrin expression ($p<0.01$). NOS inhibitor also reversed 3-nitrotyrosine-inhibited chemotaxis ($p<0.01$). Finally, 3-nitrotyrosine enhanced the expression of the inducible type of NOS ($p<0.01$) and nitric oxide release ($p<0.01$) through nuclear factor-κB activation. These results suggest that the free amino acid form of 3-nitrotyrosine can affect the tissue repair process by modulating nitric oxide production.

KEYWORDS: Inducible nitric oxide synthase, nitric oxide, reactive nitrogen species, remodelling

Excessively produced nitric oxide (NO) derived from the inducible type of NO synthase (iNOS) leads to the formation of reactive nitrogen species (RNS), including peroxynitrite and nitrogen dioxide, during inflammatory and immune processes in lung diseases [1]. These RNS are formed from NO and superoxide anions [2] or *via* the H_2O_2/peroxidase-dependent nitrite oxidation pathway [3]. Excessive RNS cause tissue injury, lipid peroxidation and nitration of tyrosine residues [1, 4]. A metabolite generally reflecting the *in vivo* production of RNS is the amino acid derivative 3-nitrotyrosine. The production of 3-nitrotyrosine has been observed in various inflammatory lung diseases, including chronic obstructive pulmonary disease [5, 6], bronchial asthma [5, 7], cystic fibrosis [8] and idiopathic pulmonary fibrosis [9].

3-Nitrotyrosine was thought to be a stable marker of RNS production [10]. However, recent studies have established that, in addition to serving as a "footprint" of RNS, the free amino acid form of 3-nitrotyrosine itself induces cytotoxity, growth inhibition and morphological changes, and consequently alters the cellular function in cultured cells [11–13]. These studies suggest that 3-nitrotyrosine may play a critical role in the tissue repair process. Indeed, its abundant production (1–120 μM) has been shown under several pathological conditions, including rheumatoid arthritis [14], liver transplantation [15], septic shock [16] and amyotrophic lateral sclerosis [17]. However, whether 3-nitrotyrosine can affect the tissue repair response remains unknown.

Inflammatory processes are frequently accompanied by alterations in the tissue structure. Such alterations may result from tissue damage due to active proteases or toxic moieties released by inflammatory cells. In addition, mediators released at inflammatory sites are capable of directly altering the cell function, leading to tissue repair and remodelling. In human lung fibroblasts, recent studies showed that cytokine stimulation is associated with iNOS gene expression [18]. Gaseous NO can also stimulate iNOS expression [19]. Furthermore, we have shown

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that cytokine-induced NO inhibited collagen gel contraction, which is a novel tissue repair model [20]. These studies suggest that iNOS expression can be stimulated in human lung fibroblasts and NO derived from iNOS may affect the tissue repair process. On the basis of these observations, we hypothesised that the free amino acid form of 3-nitrotyrosine can modulate iNOS expression in human lung fibroblasts and affect the tissue repair process.

The present study, therefore, was designed first to determine whether 3-nitrotyrosine could affect tissue remodelling through an effect on the human fetal lung fibroblast (HFL-1)-mediated contraction of collagen gels and chemotaxis towards chemoattractant. Next, we assessed whether 3-nitrotyrosine can modulate fibroblast release of fibronectin, transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF), which are thought to be critical mediators of tissue remodelling. Finally, we determined whether the effects of 3-nitrotyrosine were mediated through excessive production of NO derived from iNOS.

MATERIALS AND METHODS

Materials

Native type I collagen (rat tail tendon collagen (RTTC)) was extracted from rat tail tendons by a previously published method [21]. Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. Repeated washing with Tris-buffered saline (0.9% NaCl and 10 mM Tris, pH 7.5) was followed by dehydration and sterilisation with 50%, 75%, 95% and pure ethanol. Type I collagen was then extracted in 6 mM hydrochloric acid at 4°C. The collagen concentration was determined by weighing a lyophilised aliquot from each lot of collagen solution. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consistently demonstrated no detectable proteins other than type I collagen.

Commercially available reagents were obtained as follows: 3-nitrotyrosine, indomethacin, N^G-mono-methyl-L-arginine acetate salt (L-NMMA), sodium nitroprusside (SNP), L-N^6-(1-iminoethyl) lysine (L-NIL), 3,3',5,5'-tetramethylebenzidine, lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St Louis, MO, USA); caffeic acid phenethyl ester (CAPE) and nuclear factor (NF)-κB inhibitors were from Calbiochem (La Jolla, CA, USA); Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Invitrogen Life Technologies (Grand Island, NY, USA).

Cell culture

HFL-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ, USA) with DMEM supplemented with 10% FCS, 100 μg·mL^{-1} penicillin, 250 μg·mL^{-1} streptomycin and 2.5 μg·mL^{-1} fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 4–5 days at a 1:4 ratio. HFL-1 cells were used between the 14th and 18th passages. At 30% confluence, cells were treated with varying concentrations of 3-nitrotyrosine in DMEM containing 10% FCS for 48 h. Cells were then harvested for later assay.

Collagen gel contraction assay

Collagen gels were prepared as described previously [22, 23]. Briefly, RTTC, distilled water and 4× concentrated DMEM were combined so that the final mixture resulted in 0.75 mg·mL^{-1} collagen, with a physiological ionic strength of 1× DMEM and a pH of 7.4. Cells were plated in 100-mm dishes in DMEM containing 10% FCS and treated with or without various concentrations of 3-nitrotyrosine for 48 h. To investigate the effect of the NOS inhibitors on fibroblast-mediated gel contraction, L-NMMA or L-NIL was added to the culture media in monolayers with or without varying concentrations of 3-nitrotyrosine. The cells were trypsinised (trypsin-EDTA: 0.05% trypsin, 0.53 mM EDTA-4Na; GIBCO, Invitrogen) and suspended in serum-free DMEM. The cells were then mixed with the neutralised collagen solution so that the final cell density in the collagen solution was 3×10^5 cells·mL^{-1} and the final concentration of collagen was 0.75 mg·mL^{-1}. Aliquots (0.5 mL·well^{-1}) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon) and allowed to gel. After gelation was completed, normally within 20 min at room temperature, the gels were gently released from the 24-well tissue culture plates and transferred into 60-mm tissue culture dishes (three gels in each dish) that contained 5 mL of freshly prepared serum-free DMEM with or without various concentrations of 3-nitrotyrosine. The gels were then incubated at 37°C in a 5% CO_2 atmosphere for 3 days. To investigate the effect of L-NMMA or L-NIL on fibroblast-mediated gel contraction, L-NMMA or L-NIL was added to the culture media after the gels were released. Gel contraction was quantified daily using an Optomax V image analyser (Optomax, Burlington, MA, USA). Data were expressed as percentages of the original gel size.

Measurement of fibronectin, TGF-β1 and VEGF by ELISA

Fibronectin, TGF-β1 and VEGF in the media of the monolayer culture were determined by ELISA according to a previous report [23].

Chemotaxis assay

Cell migration was assessed using the Boyden blindwell chamber (Neuroprobe Inc., Gaithersburg, MD, USA) as previously described [23, 24]. Briefly, 26 μL of serum-free DMEM containing human fibronectin (20 μg·mL^{-1}) was placed into the bottom wells. Polycarbonate membranes with 8-μm pores (Neuroprobe Inc.), which were pre-coated with 5 μg·mL^{-1} gelatin in 0.1% acetic acid, were used. The cells were pretreated with or without various concentrations of 3-nitrotyrosine for 48 h. To investigate the role of NOS, L-NMMA was added to the culture media with or without 3-nitrotyrosine. The cells grown to 75% confluence were rinsed, re-fed with serum-free DMEM and treated with various concentrations of 3-nitrotyrosine at 37°C in a humidified atmosphere of 5% CO_2. The cells were trypsinised and suspended in serum-free DMEM at a density of 1×10^6 cells·mL^{-1}. 50 mL of cell suspension treated with various concentrations of 3-nitrotyrosine in the presence or absence of L-NMMA were then added into each top well. The cells were allowed to migrate at 37°C in a 5% CO_2 atmosphere for 6 h. Cells that had not migrated were scraped off the upper surface of the membrane and the membranes were air dried. The cells were then stained with PROTOCOL (Fisher Scientific, Swedesboro, NJ, USA) and mounted on a glass microscope slide. Chemotaxis was assessed by counting the numbers of cells in five high-power fields.

Wells with serum-free DMEM were used as negative controls and those with chemoattractant alone were used as positive controls.

Western blotting

To investigate the effects of NF-κB activation on iNOS expression, cells were treated with or without various concentrations of CAPE 30 min prior to treatment with 3-nitrotyrosine. After treatment with various concentrations of 3-nitrotyrosine for 48 h in a monolayer culture, cells were washed with 4°C PBS and homogenised in cell lysis buffer (35 mM Tris-HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 μM phenylmethylsulfonyl fluoride, 100 $\mu g \cdot mL^{-1}$ aprotinin and 1 $\mu g \cdot mL^{-1}$ leupeptin). Samples were solubilised in SDS-PAGE sample buffer. To investigate NF-κB translocation into the nucleus, cells were seeded in 60-mm dishes at a density of 1×10^5 cells$\cdot mL^{-1}$. At 90% confluence, the cells were treated with 10^{-4} M 3-nitrotyrosine. The cells were harvested at various time points. To obtain the nuclear and cytosolic fractions, a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) was used according to the manufacturer's instructions. Equal amounts of protein were loaded and separated by electrophoresis on 12.5% SDS-PAGE gels. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Primary polyclonal antibodies against human iNOS (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or $\alpha_5\beta_1$ integrin (1:1,000 dilution; Chemicon International, Inc., Temecula, CA, USA), or mouse monoclonal anti-NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) were used for detection. Bound antibodies were visualised using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) with a Typhoon Scanner (Amersham Biosciences). The intensity of the detected band was quantified by NIH image.

Quantitative PCR

Fibroblasts were treated with or without 3-nitrotyrosine and were harvested at 12 h. Total RNA was eluted using an RNeasy mini kit (Qiagen Sciences, Valencia, CA, USA). cDNA was generated using reverse transcriptase. The primers for iNOS were designed as follows: forward CCCCACGCTGCATTGG; reverse CACGTGTCTGCAGATGTGTTCA. Gene expression was measured using assays on the above probe and primers and reactions were analysed by using the ABI 7000 Taqman® system (Applied Biosystems, Foster City, CA, USA).

Measurement of nitrite/nitrate

To evaluate nitrite/nitrate production in the monolayer culture, cells were seeded in 6-well tissue plates at a cell density of 1×10^5 cells$\cdot mL^{-1}$. The cells were treated with or without varying concentrations of 3-nitrotyrosine for 48 h and the supernatant was harvested. The concentration of nitrite/nitrate was determined by a Nitric oxide assay kit (Assay Designs, Inc., Ann Arbor, MI, USA).

MTT assay

For monitoring cell viability, the 3-nitrotyrosine-treated cells were incubated with MTT solution at a final concentration of 1 $mg \cdot mL^{-1}$ for 4 h at 37°C. After incubation, dimethyl sulfoxide was added into each well. The absorbance of each sample at 570 nm was determined by a spectrophotometer using a reference wavelength of 630 nm.

Statistical analysis

Data were expressed as mean ± SEM. Multiple comparisons of experimental values among the groups were evaluated by one-way ANOVA followed by Bonferroni's *post hoc* test to test for multiple comparisons and the Mann–Whitney U-test for single comparisons. Probability values of <0.05 were considered significant.

RESULTS

At first, we examined the effect of 3-nitrotyrosine on the cell viability. 3-Nitrotyrosine did not affect the cell viability in either the collagen gel culture condition or the chemotaxis condition (see supplementary fig. 1).

To investigate the 3-nitrotyrosine modulation of collagen gels, cells cast in collagen gels were floated in media with various concentrations of 3-nitrotyrosine. 3-Nitrotyrosine significantly inhibited the gel contraction compared with control in a concentration-dependent manner (at 10^{-4} M, gel size was 81.0 ± 0.4% *versus* 65.9 ± 0.1% of initial size on day 3; $p<0.01$; fig. 1). The NOS inhibitor L-NMMA (10^{-4} M) completely abolished the inhibitory effect of 3-nitrotyrosine on gel contraction compared with the 3-nitrotyrosine-treated group (on day 3, gel size was 71.5 ± 0.7% *versus* 88.6 ± 1.2% of initial size; $p<0.01$; fig. 2a), while the cyclooxygenase inhibitor indomethacin (2×10^{-6} M) had no effect on the 3-nitrotyrosine-mediated inhibition of gel contraction (on day 3, gel size was 86.4 ± 1.0% *versus* 88.6 ± 1.2% of initial size; fig. 2b). To investigate whether iNOS was related to the L-NMMA-mediated effect, the effects of a specific iNOS inhibitor L-NIL on the 3-nitrotyrosine-mediated inhibition of gel contraction was assessed. As shown in figure 3, L-NIL significantly reversed the 3-nitrotyrosine-mediated inhibition of gel contraction in a concentration-dependent manner (at 10^{-5}–10^{-4} M; $p<0.01$). To investigate the role of iNOS in the tissue repair, we treated the cells with LPS to stimulate the iNOS expression. LPS significantly augmented iNOS expression ($p<0.01$; supplementary fig. 2a). LPS significantly attenuated fibronectin release in the media compared with control (4,217 ± 464 *versus* 8,131 ± 585 ng$\cdot$culture^{-1}; $p<0.01$; supplementary fig. 2b). L-NIL significantly restored the LPS-attenuated fibronectin release (6,750 ± 391 *versus* 4,217 ± 464 ng$\cdot$culture^{-1}; $p<0.01$; supplementary fig. 2b).

It is known that fibronectin and TGF-β1 are involved in tissue repair and are able to enhance collagen gel contraction by human lung fibroblasts. VEGF is believed to contribute to vascular cell proliferation and may be a mediator of tissue repair and remodelling. To determine whether 3-nitrotyrosine could contribute to the release of these mediators, the release of these three mediators in the monolayer culture was evaluated. 3-Nitrotyrosine (10^{-5}–10^{-4} M) significantly decreased fibronectin release in the monolayer culture ($p<0.01$; fig. 4a), while it did not affect the release of TGF-β1 (fig. 4b) and VEGF (fig. 4c) in the monolayer culture.

Because fibroblast migration from neighbouring connective tissue into sites of inflammation plays an important role in tissue repair in response to injury, we assessed the effect of

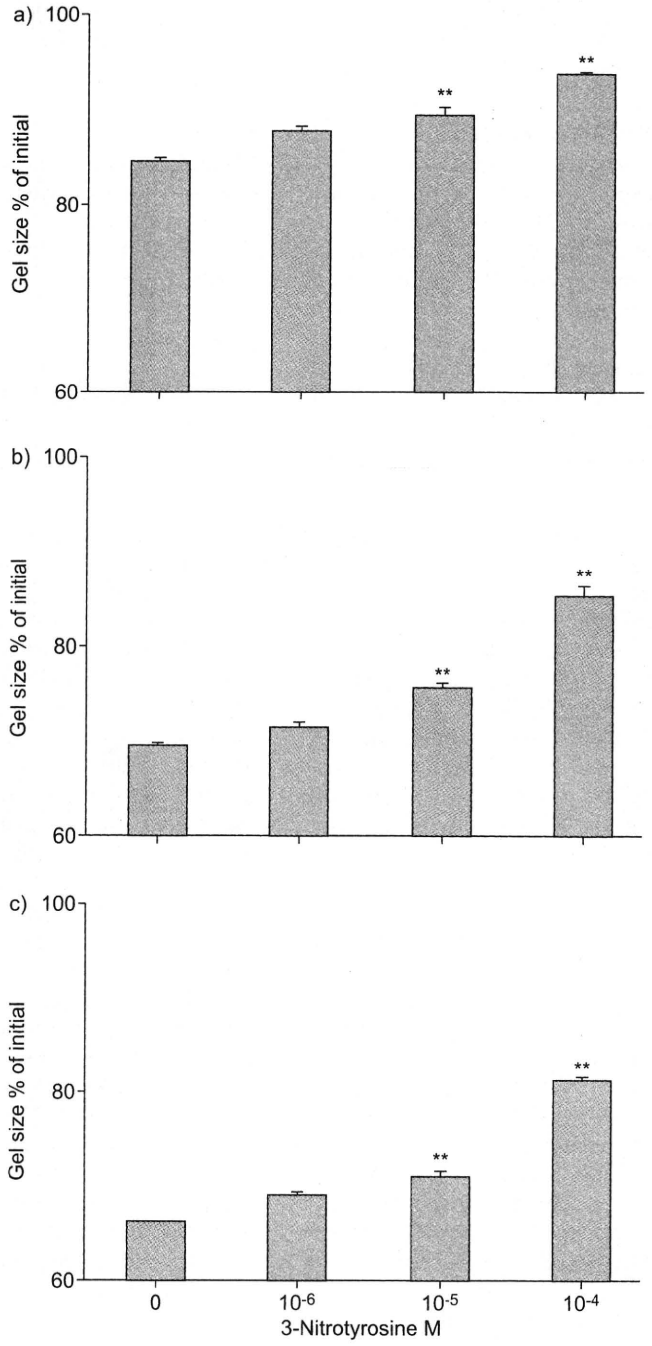

FIGURE 1. Effect of 3-nitrotyrsoine on collagen gel contraction by human fetal lung fibroblasts. Gel size was measured on a) day 1, b) day 2 and c) day 3. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control.

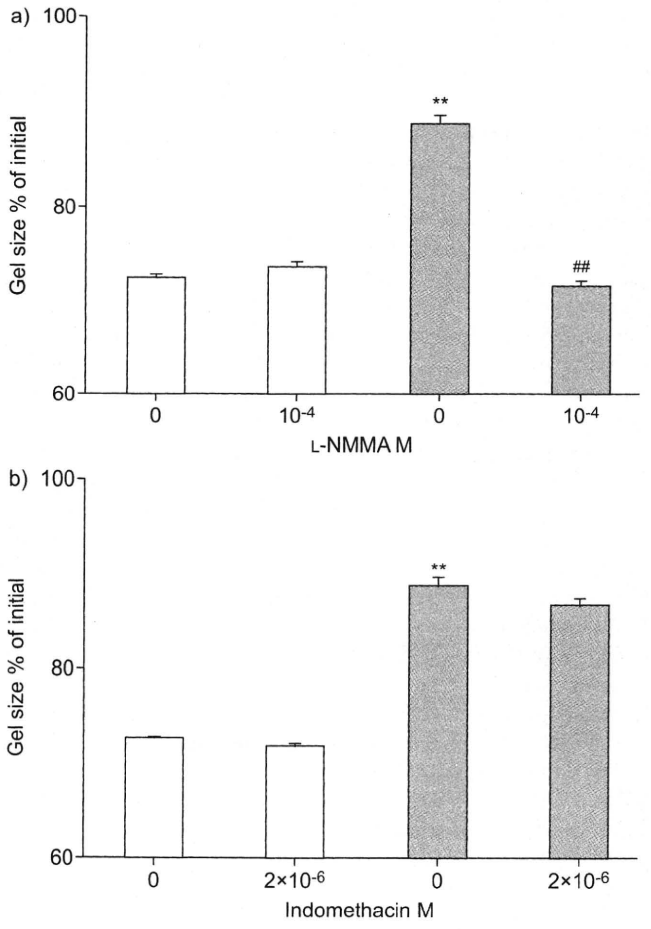

FIGURE 2. Effect of N^G-mono-methyl-L-arginine acetate salt (L-NMMA) and indomethacin on 3-nitrotyrosine-inhibited collagen gel contraction by human fetal lung fibroblasts. Fibroblasts were cast into collagen gels and maintained in floating culture in medium with (▩) or without (□) 3-nitrotyrosine at 10^{-4} M in the presence or absence of a) the nitric oxide synthase inhibitor L-NMMA or b) the cyclooxygenase inhibitor indomethacin. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with 3-nitrotyrosine-treated group.

3-nitrotyrosine on HFL-1 chemotaxis. Using fibronectin (20 $\mu g \cdot mL^{-1}$) as the chemoattractant, 3-nitrotyrosine significantly inhibited fibroblast chemotaxis compared with control in a concentration-dependent manner (at 10^{-4} M, 248±24 cells in five high power fields (HPF) *versus* 95.0±14 cells in five HPF; $p<0.01$; fig. 5a). The NOS inhibitor L-NMMA (10^{-4} M) completely abolished the 3-nitrotyrosine-mediated inhibition of chemotaxis towards fibronectin ($p<0.01$; fig. 5a). We also investigated the effect of NO on fibroblast-mediated chemotaxis. The NO donor SNP inhibited the chemotaxis in a concentration-dependent manner (at 10^{-3} M, 223±14 cells in five HPF *versus* 108±9.8 cells in five HPF; $p<0.01$; fig. 5b). To explore the possible mechanism of inhibition of chemotaxis by 3-nitrotyrosine, we investigated the effect of nitrotyrosine on the expression of $\alpha_5\beta_1$ integrin, which is a receptor for fibronectin. As shown in figure 5c, 10^{-4} M 3-nitrotyrosine decreased the expression of $\alpha_5\beta_1$ integrin (both $p<0.05$).

To clarify which mechanisms are related to the 3-nitrotyrosine-augmented iNOS expression, we investigated the effect of 3-nitrotyrosine on NF-κB activation, which is thought to be the most important pathway in the regulation of iNOS expression. Fibroblasts were incubated with 3-nitrotyrosine and examined for the translocation of NF-κB p65 into the nucleus. There was no significant change in the translocation of NF-κB into the nucleus from 0 to 120 min without 3-nitrotyrosine treatment (*e.g.* at 0 min *versus* 60 min, 1.00±0.0 *versus* 1.04±0.15 relative intensity of

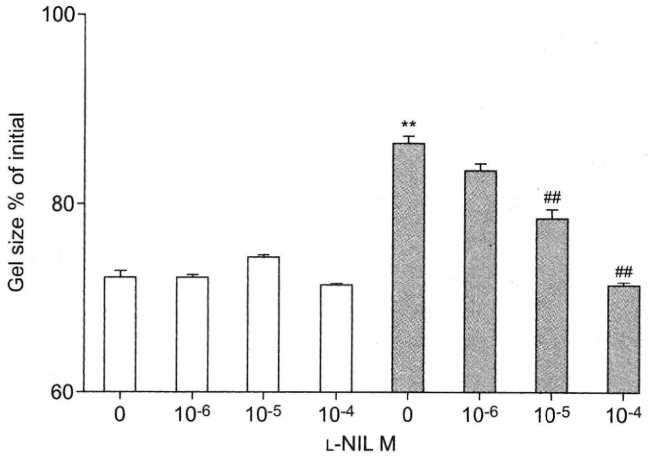

FIGURE 3. Effect of various concentrations of L-N^6-(1-iminoethyl)lysine (L-NIL) on 3-nitrotyrosine-mediated inhibition of gel contraction. Fibroblasts were maintained in floating culture in medium with (▩) or without (□) 3-nitrotyrosine at 10^{-4} M in the presence or absence of varying concentrations of L-NIL. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed group.

NF-κB/lamin A/C), whereas 10^{-4} M 3-nitrotyrosine significantly enhanced the translocation of NF-κB into the nucleus at 60 min as shown in figure 6a ($p<0.01$). Furthermore, a novel NF-κB inhibitor, CAPE, significantly suppressed the 3-nitrotyrosine-augmented iNOS expression (at 0.3–1.0 $\mu g \cdot mL^{-1}$ CAPE; $p<0.01$; fig. 6b). These results suggest that 3-nitrotyrosine stimulated NF-κB activation and augmented iNOS expression through NF-κB activation.

To clarify whether 3-nitrotyrosine augments the expression of iNOS, fibroblasts were incubated with 3-nitrotyrosine and examined for the expression of iNOS mRNA and protein. As shown in figure 7a and b, 10^{-4} M 3-nitrotyrosine significantly enhanced the expression of iNOS mRNA and protein ($p<0.01$). Furthermore, NO release in the media of the HFL-1 cell culture was investigated. 3-Nitrotyrosine significantly enhanced the release of NO in the media of the HFL-1 culture in a concentration-dependent manner (at 10^{-4} M; $p<0.01$; fig. 7c).

Because 3-nitrotyrosine is incorporated into α-tubulin in various types of cells and changes the cellular function, we investigated the incorporation of 3-nitrotyrosine into α-tubulin by western blotting. 3-Nitrotyrosine was not incorporated into α-tubulin in HFL-1 cells assessed by western blotting (supplementary fig. 3).

DISCUSSION

The present study demonstrated that the free amino acid form of 3-nitrotyrosine inhibits the fibroblast-mediated contraction of three-dimensional collagen gels and fibroblast chemotaxis towards fibronectin. 3-Nitrotyrosine also significantly decreased fibronectin release into the supernatant of the monolayer cultures. The NOS inhibitors L-NMMA and L-NIL recovered the 3-nitrotyrosine-inhibited gel contraction, mediator production and chemotaxis towards fibronectin. Furthermore, 3-nitrotyrosine enhanced the expression of iNOS protein through NF-κB activation and NO production. These results suggest that

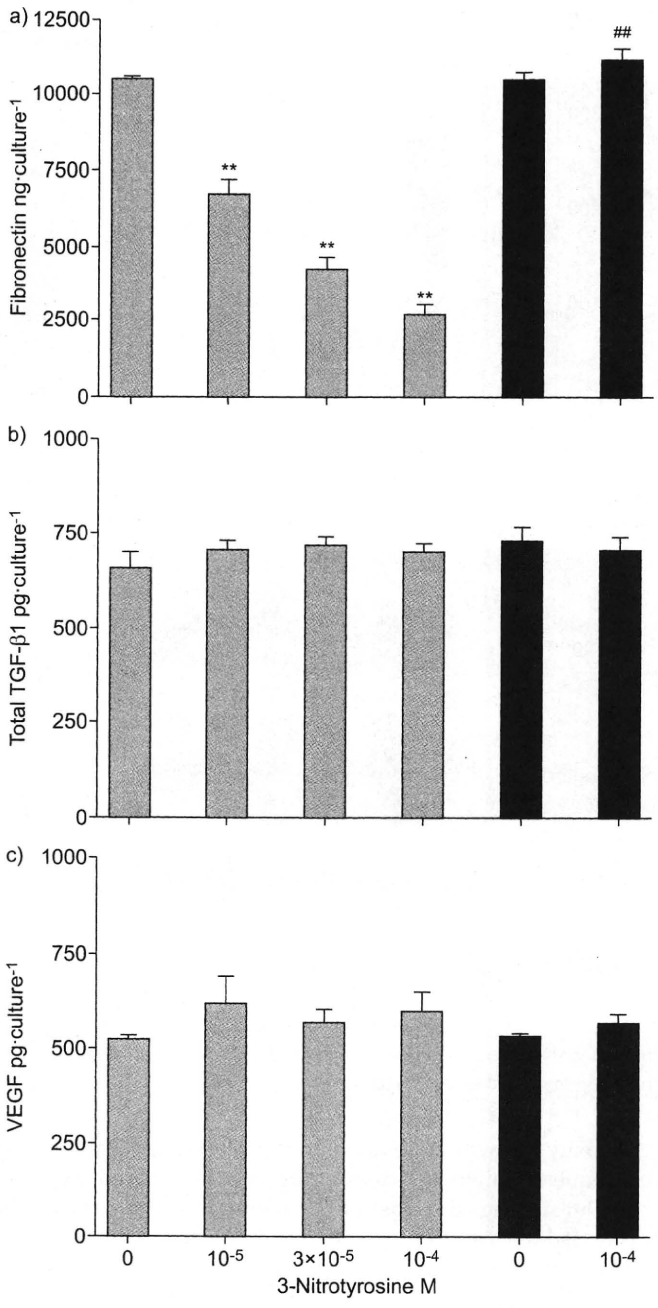

FIGURE 4. Effect of 3-nitrotyrosine on a) fibronectin, b) transforming growth factor (TGF)-β1 and c) vascular endothelial growth factor (VEGF) release. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). Media were assayed by ELISA. All values are mean±SEM for four separate experiments, each performed in duplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed (10^{-4} M) group.

3-nitrotyrosine can affect fibroblast-mediated repair processes and that the mechanism of this effect depends on the generation of NO.

3-Nitrotyrosine is a marker of nitration of the free amino acid form of tyrosine or tyrosine residues of proteins [4]. Recently, it has been reported that the free amino acid form of 3-nitrotyrosine is not only a marker of RNS, but also induces

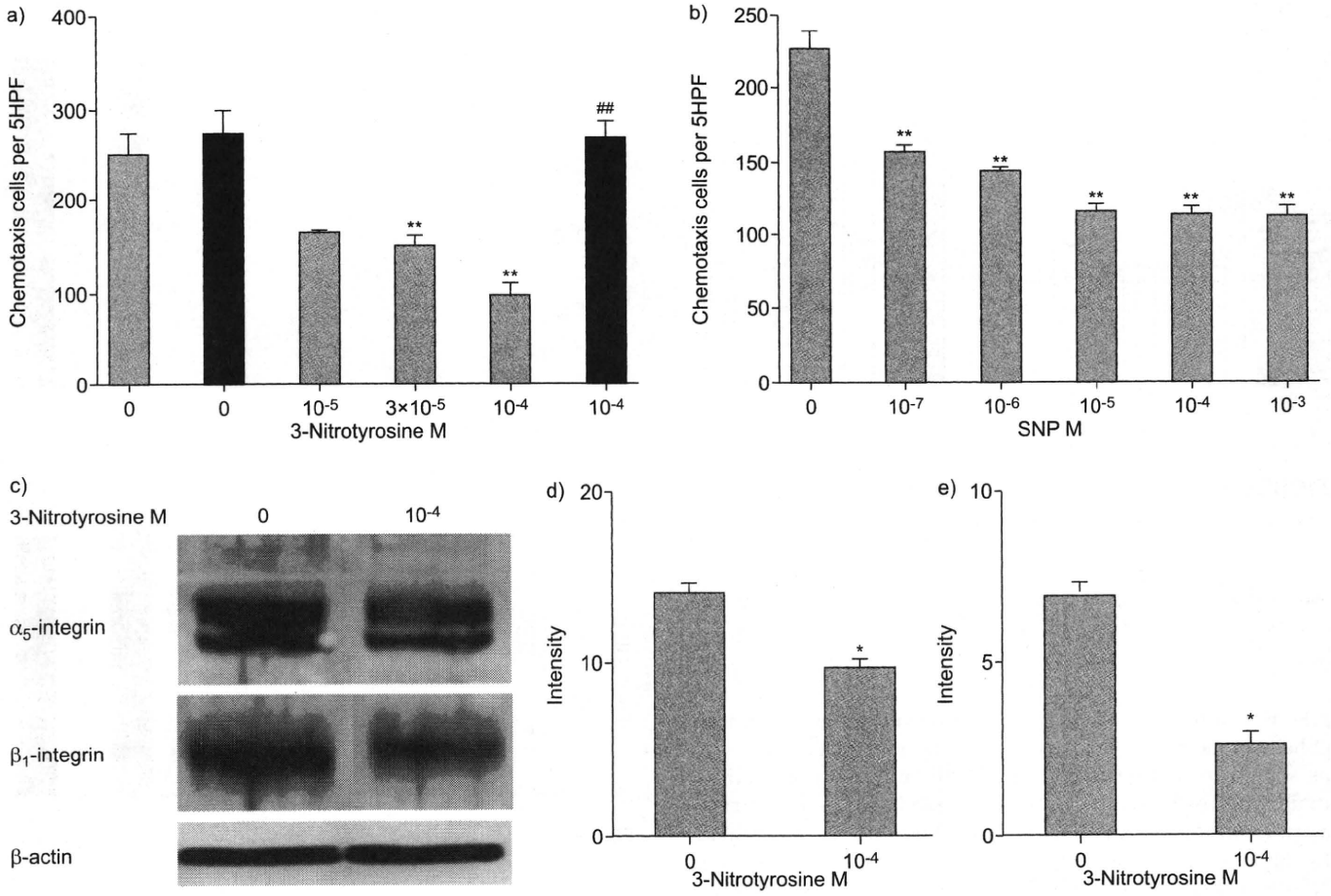

FIGURE 5. Effect of a) 3-nitrotyrosine or b) the nitric oxide donor sodium nitroprusside (SNP) on chemotaxis, and c) effect of 3-nitrotyrosine on expression of $\alpha_5\beta_1$ integrin in human fetal lung fibroblasts. Fibroblasts were exposed to various concentrations of a) 3-nitrotyrosine or b) SNP and assayed for chemotaxis towards fibronectin in the Boyden blindwell chemotaxis assay. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). c) The treated cells were analysed by western blotting for the expression of $\alpha_5\beta_1$ integrin. Band intensity was quantified by NIH image for d) α_5-integrin and e) β_1-integrin. All values are mean±SEM for four separate experiments. *: $p<0.05$ compared with control; **: $p<0.01$ compared with control; ##: $p<0.01$ compared with 3-nitrotyrosine-treated (10^{-4} M) group.

cytotoxity, growth inhibition and morphological changes, and consequently alters the cellular function in cultured cells [11–13]. Our findings demonstrate that 3-nitrotyrosine leads to the decreased release of fibronectin that drives fibroblast-mediated repair responses. Fibronectin, a multifunctional glycoprotein involved in tissue remodelling, is a chemoattractant for lung fibroblasts [25] and can be released in increased amounts from fibroblasts [25] and epithelial cells [26, 27] in response to a variety of cytokines. According to a previous study, both the cellular type and plasma type of fibronectin enhanced collagen gel contraction [28]. In the present study, 3-nitrotyrosine reduced fibronectin production and NOS inhibitor reversed the 3-nitrotyrosine-reduced fibronectin production, suggesting that NO can reduce fibronectin production in human lung fibroblast.

RNS have a variety of biological effects, including tissue injury, lipid peroxidation and nitration of protein tyrosine residues. We have shown that RNS are excessively produced in the airways of asthmatics [5] and that endogenously produced RNS cause airway inflammation in late allergic response model [29]. Recently, we have shown that peroxynitrite, one of the RNS, stimulates fibroblasts. Peroxynitrite stimulates the differentiation of fibroblasts into myofibroblasts and extracellular matrix protein production *in vitro* [23, 30], suggesting that RNS induce fibroblast-mediated profibrotic responses. In the current study, free 3-nitrotyrosine caused the inhibition of tissue remodelling *in vitro*. 3-Nitrotyrosine produced by RNS may counteract the RNS-mediated profibrotic responses.

NO is synthesised in a variety of cell types by the enzyme NOS, which exists in constitutive and inducible isoforms [29]. It has been reported that inflammatory cytokines can induce iNOS in human pulmonary fibroblasts [18]. In the current study, 3-nitrotyrosine induced iNOS protein through NF-κB activation in human lung fibroblasts. 3-Nitrotyrosine can also stimulate NO production in fibroblasts. Furthermore, the NOS inhibitor L-NMMA and the iNOS inhibitor L-NIL diminished the 3-nitrotyrosine-induced inhibition of collagen gel contraction, chemotaxis and fibronectin production, suggesting that 3-nitrotyrosine might affect the tissue repair process through NO production derived from iNOS.

The regulation of fibroblast recruitment *in vivo* is likely to depend on both the chemotactic factors and inhibitors.

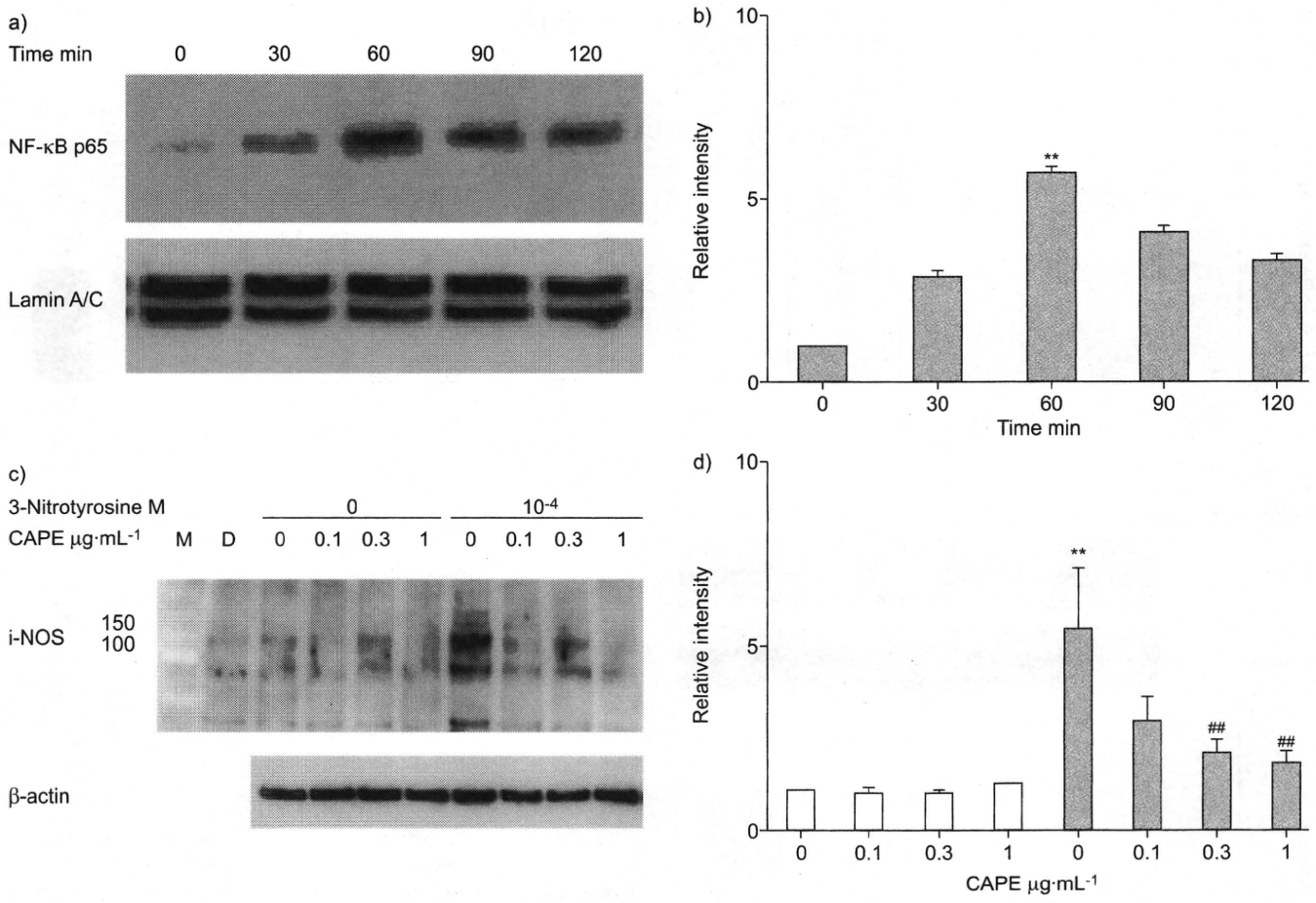

FIGURE 6. Effect of 3-nitrotyrosine on nuclear factor (NF)-κB p65 translocation into the nucleus and effect of an NF-κB inhibitor on the 3-nitrotyrosine-augmented inducible type nitric oxide synthase (iNOS) expression. a and b) Cells were treated with 10^{-4} M 3-nitrotyrosine and harvested at 0–120 min. The amount of NF-κB p65 translocated into the nucleus was assessed by western blotting. c and d) Cells were pretreated with the NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), followed by treatment with (▩) or without (□) 3-nitrotyrosine at 10^{-4} M for 48 h. Cells were harvested and iNOS expression was assessed by western blotting. b and d) Band intensity was quantified by NIH image. M: molecular marker lane; D: the same amount of control protein was loaded to prevent "smiling". All values are mean±SEM for four separate experiments. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed group.

Fibronectin can augment chemotactic activity in fibroblasts [25]. The current study demonstrated that 3-nitrotyrosine inhibited chemotaxis and NOS inhibitor abolished the 3-nitrotyrosine-induced inhibition of chemotaxis. Exogenously administrated NO also inhibited the fibroblast-mediated chemotaxis. Moreover, 3-nitrotyrosine can stimulate iNOS expression and NO production. Taken together, 3-nitrotyrosine inhibits chemotaxis through NO production. To explore the possible mechanism by which 3-nitrotyrosine inhibits fibroblast-mediated chemotaxis towards fibronectin, we investigated the effect of 3-nitrotyrosine on the expression of $\alpha_5\beta_1$ integrin, which is a receptor for fibronectin. In the present study, we found that 3-nitrotyrosine inhibited the expression of $\alpha_5\beta_1$ integrin. Therefore, 3-nitrotyrosine may inhibit chemotaxis through the suppression of $\alpha_5\beta_1$ integrin expression.

3-Nitrotyrosine could stimulate iNOS protein expression and NO production in the current study. Interestingly, 3-nitrotyrosine can stimulate NF-κB translocation into the nucleus. In general, NF-κB activation is a key process in the regulation of iNOS expression. To our knowledge, this is the first report showing that 3-nitrotyrosine stimulates NF-κB activation in lung fibroblasts. Although RNS are reported to stimulate NF-κB [30], nitrotyrosine derived from RNS may cause inflammation through NF-κB.

In the current study, we attempted to examine the role of NO derived from all types of NOS in the fibroblast function. We found that NO could attenuate the fibroblast function because a nonspecific NOS inhibitor, L-NMMA, restored the 3-nitrotyrosine-inhibited gel contraction (fig. 2a). Then, we found that 3-nitrotyrosine augmented iNOS expression through NF-κB activation. In the next step, we investigated the effects of NO derived from iNOS on the fibroblast function by means of a specific iNOS inhibitor, L-NIL. As we expected, the iNOS inhibitor significantly restored the 3-nitrotyrosine-inhibited gel contraction to the same degree as L-NMMA, as shown in figures 2a and 3, suggesting that NO derived from iNOS is a key mediator in the attenuation of the fibroblast function. Although we only showed that NO derived from iNOS could inhibit the gel contraction in the present study, these findings encourage us to speculate that L-NIL also may inhibit other fibroblast functions, including mediator production, chemotaxis and NO release.

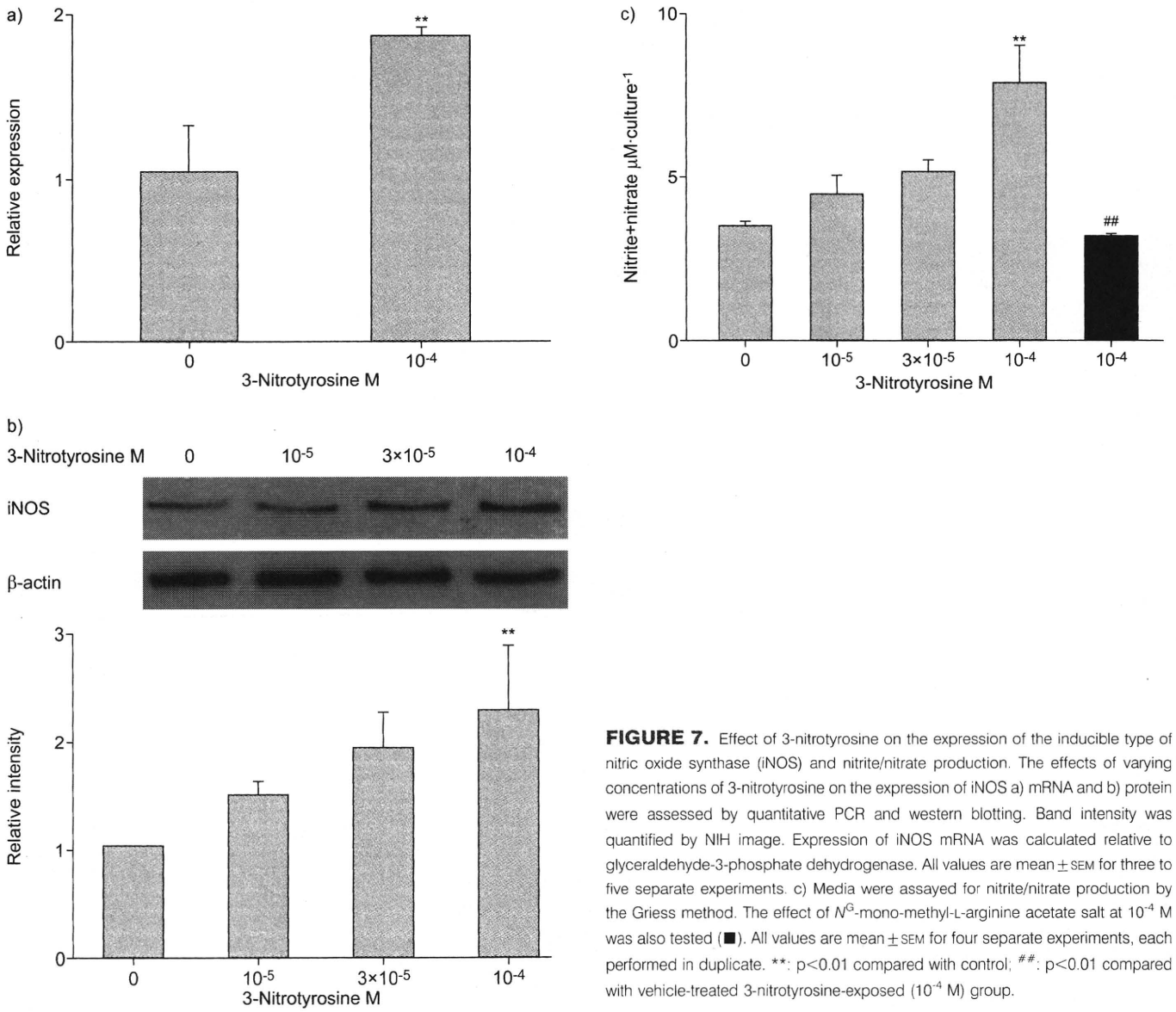

FIGURE 7. Effect of 3-nitrotyrosine on the expression of the inducible type of nitric oxide synthase (iNOS) and nitrite/nitrate production. The effects of varying concentrations of 3-nitrotyrosine on the expression of iNOS a) mRNA and b) protein were assessed by quantitative PCR and western blotting. Band intensity was quantified by NIH image. Expression of iNOS mRNA was calculated relative to glyceraldehyde-3-phosphate dehydrogenase. All values are mean ± SEM for three to five separate experiments. c) Media were assayed for nitrite/nitrate production by the Griess method. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). All values are mean ± SEM for four separate experiments, each performed in duplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed (10^{-4} M) group.

It has been reported that 3-nitrotyrosine is incorporated into α-tubulin, induces cell morphology transformation and changes the cell function [13]. Therefore, we investigated whether 3-nirotyrosine attenuates the tissue repair because of the incorporation of 3-nitrotyrosine into α-tubulin. To clarify this, we investigated the incorporation of 3-nitrotyrosine into α-tubulin by western blotting. 3-Nitrotyrosine was not incorporated into the cells (supplementary fig. 3). However, we could not confirm whether the incorporation of 3-nitrotyrosine into α-tubulin is associated with the inhibition of tissue repair observed in the current study because of the lack of a specific tubulin tyrosine ligase inhibitor. The findings that 3-nitrotyrosine is not incorporated into the cells and that NOS inhibitors inhibited the 3-nitrotyrosine-mediated inhibition of tissue repair suggest that the 3-nitrotyrosine incorporation may have had little influence on our current findings.

In summary, our data demonstrate that 3-nitrotyrsine inhibited the fibroblast-mediated contraction of three-dimensional collagen gels, chemotaxis and fibronectin production. 3-Nitrotyrosine also augmented iNOS protein expression through NF-κB activation and the release of NO. These effects of 3-nitrotyrosine were significantly blocked by NOS inhibitor. These results suggest that the free amino acid form of 3-nitrotyrosine can affect the tissue repair process by modulating NO production.

STATEMENT OF INTEREST

A statement of interest for S.I. Rennard can be found at www.erj.ersjournals.com/misc/statements.dtl

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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).

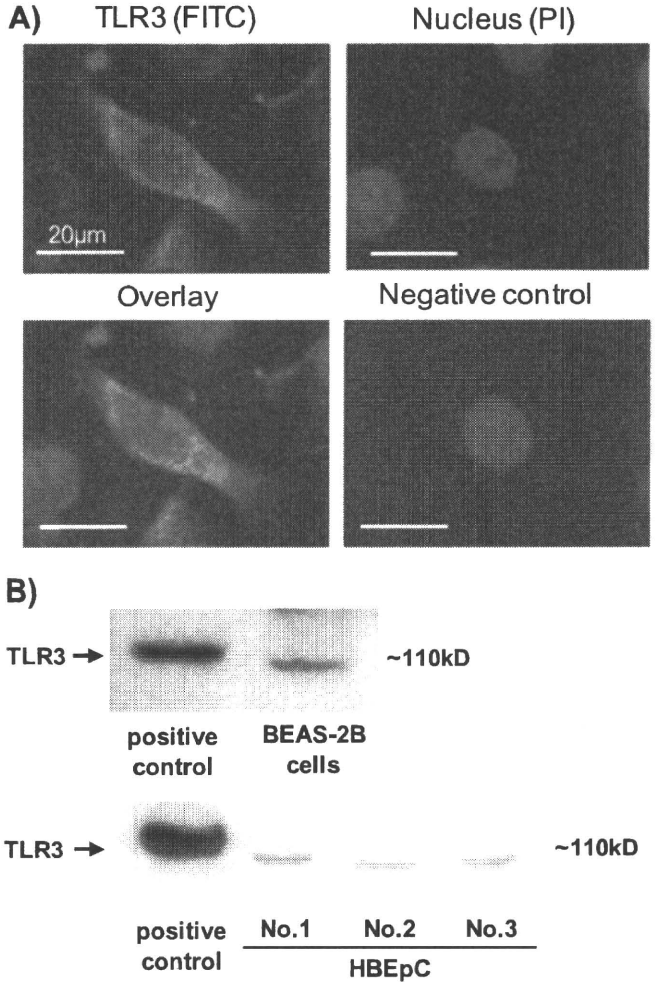

Figure 1. Expression of Toll-like receptor 3 (TLR3) in human bronchial epithelial cells. (*A*) Panels show representative photographs of immunoreactivity of TLR3 in BEAS-2B cells (*upper left panel, green*), immunofluorescence of the nucleus (*upper right panel, red*), an overlaid image (*lower left 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

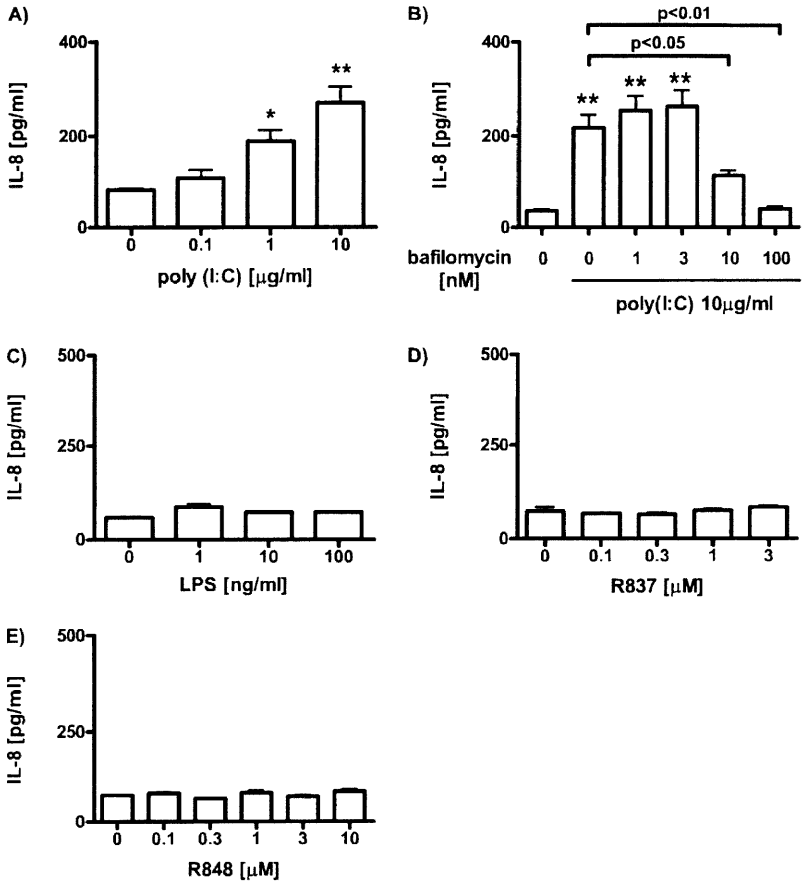

Figure 2. Effect of ligands for TLRs on IL-8 release and effect of bafilomycin on poly(I:C)-induced IL-8 release in BEAS-2B cells. (*A*) BEAS-2B cells were treated with various concentrations of poly(I:C). (*B*) The cells were treated with 10 μg/ml poly(I:C) or vehicle in the presence of various concentrations 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.