Original Research

Neutrophil elastase inhibitor reduces ventilation-induced lung injury via nuclear factor-κB and NF-κB repressing factor in mice

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Abstract

Mechanical ventilation used in patients with acute lung injury can damage pulmonary epithelial cells through production of inflammatory cytokines, oxygen radicals, and neutrophil infiltration, termed ventilator-induced lung injury. Neutrophil elastase, nuclear factor-κB (NF-κB), and NF-κB repressing factor (NRF) have previously been shown to participate in the regulation of macrophage inflammatory protein-2 (MIP-2) during airway inflammation. However, the mechanisms regulating interactions among mechanical ventilation, neutrophil influx, and NF-κB/NRF remain unclear. Thus, we hypothesized that neutrophil elastase inhibitor attenuated ventilation-induced neutrophil recruitment and MIP-2 production through inhibition of the NF-κB/NRF pathway. Male C57BL/6 mice were exposed to low-tidal-volume (6 mL/kg) or high-tidal-volume (30 mL/kg) mechanical ventilation using room air with or without 2 μg/g NF-κB inhibitor SN50 or 6 μg/g NRF short interfering RNA or 100 μg/g neutrophil elastase inhibitor administration. Nonventilated mice served as a control group. Evan blue dye, lung wet-to-dry weight ratio, free radicals, myeloperoxidase, histopathologic grading of lung tissue, inflammatory cytokines, Western blot of NF-κB and NRF, and gene expression of NRF were measured to establish the extent of lung injury. Neutrophil elastase inhibitor ameliorated high-tidal-volume ventilation-induced lung injury, neutrophil influx, production of MIP-2 and malondialdehyde, activation of NF-κB and NRF, apoptotic epithelial cell death, and disruption of bronchial microstructure in mice. Mechanical stretch-augmented acute lung injury was also attenuated through pharmacological inhibition of NF-κB activity by SN50 and NRF expression by NRF short interfering RNA. Our data suggest that neutrophil elastase inhibitor attenuates high-tidal-volume mechanical ventilation-induced neutrophil influx, oxidative stress, and production of MIP-2, at least partly, through inhibition of NF-κB/NRF pathway. Understanding the protective effects of neutrophil elastase inhibitor associated with the reduction of MIP-2 allow clarification of the pathophysiological mechanisms regulating severe lung inflammation and development of possible therapeutic strategies involved in acute lung injury.

Keywords: Acute respiratory distress syndrome, neutrophils, ventilator-induced lung injury

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are characterized by interstitial pulmonary edema, respiratory distress, and hypoxemia.[1] High-tidal-volume mechanical ventilation in patients with ARDS has been shown to increase the risk of pathologic overdistention in the lungs, elicit the production of proinflammatory cytokines, neutrophil influx, apoptotic cell death, and eventually induce a type of ALI, termed ventilator-induced lung injury (VILI).[2–5] The neutrophil accumulation in the lungs during ALI leads to the production of inflammatory mediators, including neutrophil elastase, reactive oxygen species (ROS), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-α (TNF-α), and interleukin-β (IL-1β), and demonstrates increased activation of the transcriptional factors, including nuclear factor-κB (NF-κB) and NF-κB repressing factor (NRF).[5–9]

NF-κB is a pivotal regulator for many inflammatory mediators involved in the pathogenesis of ALI.[10–12] A previous study of patients with sepsis-induced ALI, elevations in the levels of NF-κB were associated with worse survival in critically ill patients.[11] In an experimental mouse model of VILI, inhibition of NF-κB activation ameliorated the severity of lung injury.[12] Notably, neutrophil elastase, a serine protease produced by neutrophils, was shown to induce IL-8 gene transcription via NF-κB activation in a lung epithelial cell line.[13] Some investigators also demonstrate that inhibition of neutrophil elastase is crucial in the protection of lung from ischemia-reperfusion injury and endotoxemia-induced injury.[7] In almost all cases, NF-κB does not act alone to enhance promoter activity.[11] Instead, p65 often physically associates with other DNA-binding factors and cooperatively acts to regulate transcription of target genes.[14]

NRF is a constitutively expressed nuclear transcription factor that binds to the negative regulatory element (NRE) in the beta interferon (IFN-β), IL-8, inducible nitric oxide synthase (iNOS), HIV type I (HIV-1) promoters, and represses the basal transcription of these genes.[14–17] Recently, NRF was found to act together with NF-κB p65 to stimulate the IL-8 gene expression in IL-1 treated epithelial cervical cells.[14,18] However, the precise mechanisms of NF-κB/NRF pathway involved in mechanical stretch-induced ALI remain unclear. To determine the roles of neutrophils, neutrophil elastase, NF-κB, and NRF in VILI, we explored how high lung stretch induced MIP-2 production. We hypothesized that neutrophil elastase inhibitor would decrease neutrophil infiltration, lung edema, production of free radicals, and MIP-2 production in mice exposed to high-tidal-volume ventilation via inhibiting NF-κB/NRF pathway. In the high-tidal-volume ventilation-induced ALI model in mice, we compared the effects among different tidal volume of mechanical ventilation, correlation of ALI to neutrophil influx, and production of MIP-2 using short interfering RNA (siRNA) targeted to NRF and with pharmacological inhibitor of NF-κB, SN50.[19]

Materials and methods

Experimental animals

Male C57BL/6 mice, aged between 6 and 8 weeks, weighing between 20 and 25 g, were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were distributed into eight groups and five mice per group in each experiment. The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Permit Number: 2011093005). All surgeries were performed under ketamine and xylazine anesthesia and all efforts were made to minimize suffering.

Ventilator protocol

We used our established mouse model of VILI as previously described.[3] In brief, tracheostomy was performed under general anesthesia with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) followed by ketamine (0.1 mg/g/h) and xylazine (0.01 mg/g/h) at a rate of 0.09 mL/10g/h by a continuous intraperitoneal infusion. The mice were placed in a supine position on a heating blanket and then attached to a Harvard apparatus ventilator, model 55-7058 (Harvard Apparatus, Holliston, MA, USA), set to deliver either 6 mL/kg at a rate of 135 breaths per min or 30 mL/kg at a rate of 65 breaths per min, for 1 to 4 h while breathing room air with zero end-expiratory pressure. The tidal volume delivered by the ventilator was checked by fluid displacement from an inverted calibration cylinder. Continuous monitoring of end-tidal CO_2 by a microcapnograph (Columbus Instruments, Columbus, OH, USA) was performed, and respiratory frequencies of 135 breaths per min for 6 mL/kg and 65 breaths per min for 30 mL/kg were chosen in the experiment, with end-tidal CO_2 at 30 to 40 mm Hg. Airway peak inspiratory pressure was measured with a pressure-transducer amplifier (Gould Instrument Systems, Valley View, OH, USA) connected to the tubing at the proximal end of the tracheostomy. Mean arterial pressure was monitored every hour during mechanical ventilation by using the same pressure-transducer amplifier connected to a 0.61 mm outer diameter (0.28 mm inner diameter) polyethylene catheter ending in the common carotid artery. One hour of mechanical ventilation for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot and 4 h for MIP-2 production, cell counts, lung water, Evans blue dye (EBD) assay, myeloperoxidase (MPO), free radicals, electron microscopy, and histopathologic staining were used based on previous studies.[3] Control, nonventilated mice were supplied with 0.09 mL/10 g/h saline intraperitoneally, and sacrificed at the end of study. At the end of the study period, heparinized blood was taken from the arterial line for analysis of arterial blood gas, and the mice were sacrificed.

Neutrophil elastase activity

Neutrophil elastase activity in bronchoalveolar lavage fluid was determined spectrophotometrically using N-methoxy-succinyl-Ala-Ala-Pro-Val p-nitroanilide, a highly specific synthetic substrate for neutrophil elastase (Calbiochem, San Diego, CA, USA).[7] Briefly, 70 μL of bronchoalveolar lavage fluid was incubated with 200 μL of reaction buffer (0.45 M Tris-HCl and 2 M NaCl, pH 8.0) and 30 μL of 0.2 mM elastase substrate I (Calbiochem) at 37°C for 24 h and the amount of liberated p-nitroanilide was determined spectrophotometrically at 410 nm. Neutrophil elastase activity was calculated from the standard curve of *p*-nitroanilide absorbance.

Short interfering RNA administration

A single dosage of 6 μg/g of targeting NRF Stealth siRNA or non-targeting Stealth siRNA (scrambled siRNA, Invitrogen, Carlsbad, CA, USA) in lipofectamine RNAiMAX was given intratracheally 48 h before mechanical ventilation.[20,21] The dosage was chosen on the basis of our previous dose–response studies that showed 6 μg/g inhibited NRF activity.[2]

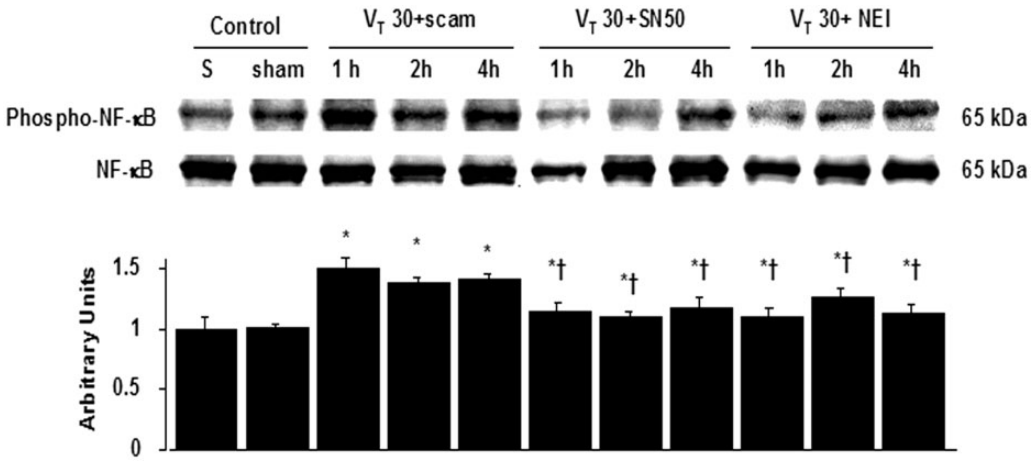

Figure 1 SN50 and neutrophil elastase inhibitor reduced lung stretch-induced NF-κB phosphorylation. Western blot was performed using an antibody which recognizes the phosphorylated NF-κB expression and an antibody that recognizes NF-κB protein expression from the lungs of control, nonventilated mice and mice ventilated with V_T 30 mL/kg (V_T30) breathing room air at indicated time periods. SN50 (2 μg/g) or NEI (100 μg/g) was given intraperitoneally 30 min before mechanical ventilation. Data shown here are representative results of five independent experiments. Arbitrary units are expressed as the ratio of phospho-NF-κB to NF-κB. *$P < 0.05$ vs. Nonventilated control treated with normal saline, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. NEI: neutrophil elastase inhibitor; NF-κB: nuclear factor-κB; S: normal saline; Sham: nonventilated control treated with nontargeting scrambled siRNA; Scam: nontargeting scrambled siRNA; siRNA: short interfering RNA

Pharmacological inhibitor

NF-κB inhibitor (SN-50, Calbiochem) 2 μg/g was given intraperitoneally 30 min before ventilation based on our present and previous studies that showed 2 μg/g inhibited NF-κB activity (Figure 1). Neutrophil elastase inhibitor (Sivelestat sodium hydrate, Sigma Chemical, St. Louis, MO) 100 μg/g was given intraperitoneally 30 min before ventilation based on previous *in vivo* studies that showed 100 μg/g inhibited neutrophil elastase activity.[7]

Immunoblot analysis

The lungs were homogenized in 3 mL of lysis buffer (20 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, 50 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiotreitol, 400 μM aprotinin, and 400 μM phenylmethylsulfonyl fluoride), transferred to eppendorff tubes and placed on ice for 15 min. Tubes were centrifuged at 14,000 rpm for 10 min at 4°C and supernatant was flash frozen. Crude cell lysates were matched for protein concentration, resolved on a 10% bis-acrylamide gel, and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). For assay of NF-κB phosphorylation and total NF-κB and NRF protein expression, Western blot analyses were performed with antibodies of phospho-NF-κB, NF-κB, and NRF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed by enhanced chemiluminescence (NEN Life Science Products, Boston, MA, USA).

Reverse transcription-polymerase chain reaction

For isolating total RNA, the lung tissues were homogenized in TRIzol reagents (Invitrogen Corporation) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed by using a GeneAmp PCR system 9600 (PerkinElmer, Life Sciences, Inc., Boston, MA, USA), as previously described.[2] The following primers were used for PCR: NRF, forward primer 5′-GTTCTGCCAAA CACTGGACC-3′ and reverse primer 5′-CTGAGATAGGCTCCCGTATGCCC-3′ and GAPDH as internal control, forward primer 5′-AATGCATCCTGCA CCACCAA-3′ and reverse primer 5′-GTAGCCATAT TCATTGTCATA-3′ (Integrated DNA Technologies, Inc., Coralville, IA).[22]

Measurement of macrophage inflammatory protein-2

At the end of the study period, the lungs were lavaged via tracheostomy with 20-gauge angiocatheter (sham instillation) three times with 0.6 mL of 0.9% normal saline. The effluents were pooled and centrifuged at 2000 rpm for 10 min. Supernatants were frozen at −80°C for further analysis of the cytokine. MIP-2 with a lower detection limit of 1 pg/mL was measured in bronchoalveolar lavage fluid using a commercially available immunoassay kit containing primary polyclonal anti-mouse antibody that was cross-reactive with rat and mouse MIP-2 (Biosource International, Camarillo, CA, USA). Each sample was run in duplicate according to the manufacturer's instructions.

Histopathologic grading of VILI

The lung tissues from control, nonventilated mice and mice exposed to high tidal volume ventilation for 4 h while breathing room air were removed *en bloc* and filled with 10% neutral buffered formalin (pH 6.8 to 7.2) at 30 cm H_2O pressure via polyethylene tubing inserted into the trachea. The lungs were paraffin embedded, sliced at 4 μm, stained with hematoxylin and eosin, and reviewed from 10 nonoverlapping fields by a single investigator blinded to therapeutic category of the mouse. Lung injury was scored using an average score of the following

items: alveolar congestion, hemorrhage, infiltration of neutrophils into airspace or the vessel wall, and thickness of the alveolar wall.[2] A score of 0 represented normal lungs: 1, mild, <25% lung involvement; 2, moderate, 25% to 50% lung involvement; 3, severe, 50% to 75% lung involvement; and 4, very severe, >75% lung involvement.

Immunohistochemistry

The lungs were paraffin embedded, sliced at 4 μm, deparaffinized, antigen unmasked in 10 mM sodium citrate (pH mM sodium citrate (pH 6.0), incubated with goat NRF or rabbit phospho-NF-κB primary antibody (1:100; Santa Cruz Biotechnology), and biotinylated goat anti-rabbit secondary antibody (1:100) according to the manufacturer's instruction for an immunohistochemical kit (Santa Cruz Biotechnology).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assay

The lung tissues from control, nonventilated mice, mice exposed to low tidal volume, and mice receiving high tidal volume ventilation for 4 h while breathing room air were paraffin embedded, sliced at 4 μm, and stained with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) reaction mixture using Fluorescein-FragEL™ DNA Fragmentation detection kit according to the manufacturer's instructions (Oncogen Research Products, Boston, MA, USA). The specimens were further examined with the Leica TCS 4D confocal laser scanning microscopy system (Leica, Wetzlar, Germany). A bright green signal indicated positive staining of apoptotic cells while shades of dull green signified nonreactive cells. Apoptosis-positive cells were quantified as the average number of epithelial cells with bright green signals per bronchiole, which were counted from 10 nonoverlapping fields by a single investigator blinded to therapeutic category of the mouse.

Transmission electron microscopy

The lungs were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C. The lungs were then postfixed in 1% osmium tetroxide (pH 7.4), dehydrated in a graded series of ethanol and embedded in EPON-812. Thin sections (70 nm) were cut, stained with uranyl acetate and lead citrate and examined on a Hitachi H-7500 EM transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

Statistical evaluation

The Western blots and NRF mRNA were quantitated using a National Institutes of Health (NIH) image analyzer, ImageJ 1.27 z (National Institute of Health, Bethesda, MD, USA), and presented as arbitrary units. Values were expressed as the mean ± SD for at least five experiments. The data of EBD assay, lung wet-to-dry weight ratio, MIP-2, neutrophil elastase activity, MPO, immunohistochemical assay, and malondialdehyde (MDA), TUNEL assay were conducted by using Statview 5.0 (Abascus Concepts Inc. Cary, NC, USA; SAS Institute, Inc.). All results of Western blots and NRF mRNA were normalized to control, nonventilated mice with room air. ANOVA was used to assess the statistical significance of the differences, followed by multiple comparisons with a Scheffe's test, and a $P < 0.05$ was considered statistically significant. Regression coefficients were calculated using the simple regression test in Statview.

EBD analysis, analysis of lung water, cell counts, MPO assay, and measurement of MDA were performed as previously described.[2,3]

Results

Reduced lung stretch-induced NF-κB phosphorylation by SN50 and neutrophil elastase inhibitor

NF-κB and NRF have been shown to modulate the neutrophil activation involved in airway inflammation.[5,8] Previous *in vivo* studies of ALI in mice have demonstrated that intranasal or intratracheal delivery of siRNA with lipofectamine ameliorated lung injury.[20,21] Furthermore, we have recently revealed that NRF siRNA decreased the expression of NRF in a dose- and time-dependent manner.[2] Moreover, we measured the activity of NF-κB from 1 to 4 h of high-tidal-volume (V_T30 mL/kg; denoted as V_T30) mechanical ventilation with or without administration of either SN50 or neutrophil elastase inhibitor to determine the time course of stretch-induced NF-κB phosphorylation and the effectiveness of SN50 and neutrophil elastase inhibitor (Figure 1). There were time-dependent increases in the phosphorylation of NF-κB, but there were no significant changes in the expression of total nonphosphorylated proteins of NF-κB. The increased activation of NF-κB was reduced by pharmacological inhibition with either SN50 or neutrophil elastase inhibitor and remained effective after 4 h of mechanical ventilation (Figure 1).

Improvement of VILI by neutrophil elastase inhibitor

We employed high-tidal-volume mechanical ventilation with ambient air for 4 h to induce VILI in male C57BL/6 mice and examined the treatment effects of neutrophil elastase inhibitor, SN50, and NRF siRNA individually or combined (Figure 2). Physiological conditions at the beginning and end of ventilation were shown in Table 1. Histological examinations revealed that the animal lungs injured by mechanical ventilation at V_T30, but not at low-tidal-volume (V_T6 mL/kg, denoted as V_T6), displayed a pattern of alveolar congestion, hemorrhaging, thickening of the alveolar wall, and neutrophil infiltration, which were largely attenuated by the administration of neutrophil elastase inhibitor, SN50, or NRF siRNA (Figure 2a). The lung injury score quantification confirmed the V_T30-induced severe damage and the therapeutic potential of neutrophil elastase inhibitor, SN50, or NRF siRNA (Figure 2b). A V_T30 also increased lung Evans blue dye content and the wet-to-dry ratio, indicating capillary leakage. However, a V_T6 showed no effect on these parameters when compared with nonventilated mice (Figure 2(c) and (d)). The microscopic lung injury and elevation of capillary permeability induced by a V_T30 was substantially suppressed by treatment with neutrophil elastase inhibitor, SN50, or NRF siRNA (Figure 2(b)

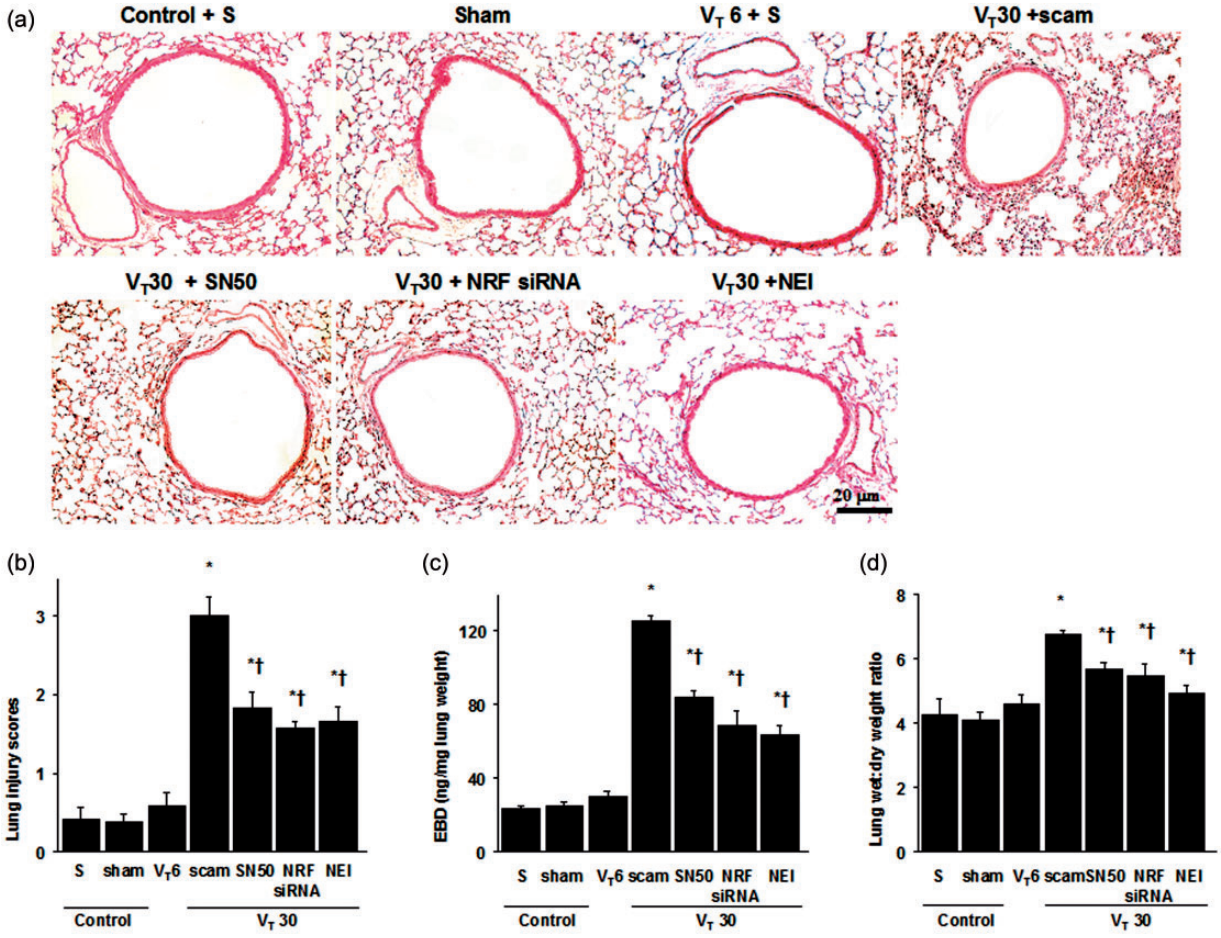

Figure 2 SN50, NRF siRNA, or neutrophil elastase inhibitor reduced lung stretch-induced lung edema and injury. (a) Histological examination and (b) quantification of high-tidal-volume-induced airway structural damage and the effects of SN50, NRF siRNA, or NEI. The effects of administering SN-50, NRF siRNA, or NEI on (c) lung EBD, and (d) the wet-to-dry ratio in wild-type mice receiving mechanical ventilation at a low-tidal-volume (V_T6) or a high-tidal-volume (V_T30) are shown. NRF siRNA, 6 μg/g, was given intratracheally 48 h before mechanical ventilation. Data shown here are the mean ± SD of five independent experiments. In panels (b), (c), and (d), *$P < 0.05$ vs. nonventilated control treated with normal saline or mice receiving a V_T 6 mL/kg, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. Scale bars represent 20 μm. EBD: Evans blue dye; NRF: NF-κB repressing factor. (A color version of this figure is available in the online journal.)

Table 1 Physiologic conditions at the beginning and end of ventilation

	Control saline	V_T 6 mL/kg saline	V_T 30 mL/kg scam	V_T 30 mL/kg SN50	V_T 30 mL/kg NRF siRNA	V_T 30 mL/kg NEI
PH	7.40 ± 0.07	7.39 ± 0.08	7.35 ± 0.04	7.36 ± 0.08	7.37 ± 0.08	7.36 ± 0.05
PaO_2 (mmHg)	95.0 ± 4.9	93.4 ± 4.1*†	86.8 ± 5.1*	91.3 ± 5.0*†	92.4 ± 5.7*†	91.9 ± 5.1*†
PaO_2/FiO_2 ratio	452.5 ± 4.4	444.8 ± 9.0*†	413.1 ± 4.9*	434.6 ± 4.2*†	440.2 ± 7.3*†	437.5 ± 9.1*†
$PaCO_2$ (mmHg)	39.1 ± 0.2	41.2 ± 0.4	36.1 ± 1.0	36.9 ± 1.2	37.1 ± 0.4	36.7 ± 1.1
MAP (mmHg)						
Start	84 ± 1.1	83.3 ± 1.5	82.9 ± 1.4	82.5 ± 1.5	82.1 ± 0.7	82.3 ± 1.7
End	82 ± 0.5	80.9 ± 1.7	76.8 ± 4.6	77.6 ± 3.9	78.5 ± 0.6	77.9 ± 2.6
PIP, mm Hg						
Start		9.9 ± 1.2	23.4 ± 1.2	23.6 ± 1.4	23.5 ± 1.2	23.7 ± 1.8
End		11.2 ± 0.8	26.8 ± 1.9	25.8 ± 2.6	25.7 ± 1.8	25.9 ± 2.1

Note: At the end of the study period, arterial blood gases and mean arterial pressure were obtained from nonventilated mice and mice ventilated at a tidal volume of 6 mL/kg or 30 mL/kg for 4 h ($n = 10$ per group). The physiological data of control groups were similar during the experiment and were used as the beginning data of ventilation. The normovolemic statuses of mice were maintained by monitoring mean artery pressure. Because no differences were observed between control, nonventilated mice with or without nontargeting scrambled siRNA, the data were combined.

FiO_2: fraction of inspired oxygen; MAP: mean arterial pressure; NRF siRNA: nuclear factor-κB repressing factor short interfering RNA; PIP: peak inspiratory pressure; SN50: NF-κB inhibitor; NEI: neutrophil elastase inhibitor; Scam: nontargeting scrambled siRNA; V_T: tidal volume.

*$P < 0.05$ vs. nonventilated control treated with normal saline.

†$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA.

to (d)). Administration of both neutrophil elastase inhibitor and SN50 reduced microvascular permeability more significantly (the lung injury score quantification: V_T 30 mL/kg with neutrophil elastase inhibitor = 1.7 ± 0.2 versus V_T 30 mL/kg with neutrophil elastase inhibitor and SN50 = 1.3 ± 0.1, $P = 0.01$; levels of EBD: V_T 30 mL/kg with neutrophil elastase inhibitor = 62.7 ± 5.2 ng/mg lung weight versus V_T 30 mL/kg with neutrophil elastase inhibitor and SN50 = 52.8 ± 4.5 ng/mg lung weight, $P = 0.01$; wet-to-dry weight ratio: V_T 30 mL/kg with neutrophil elastase inhibitor = 4.9 ± 0.3 versus V_T 30 mL/kg with neutrophil elastase inhibitor and SN50 = 4.3 ± 0.2, $P = 0.02$; levels of MIP-2: V_T 30 mL/kg with neutrophil elastase inhibitor = 20.1 ± 0.6 pg/mL BAL versus V_T 30 mL/kg with neutrophil elastase inhibitor and SN50 = 17.2 ± 0.4 pg/mL BAL, $P = 0.03$). Therefore, these data suggest that neutrophil elastase inhibitor, SN50, or NRF siRNA improve microvascular leakage, lung edema, and total lung injury in a VILI model induced by a V_T30. There were no statistically significant differences between EBD and wet-to-dry ratio in mice receiving mechanical ventilation at V_T 30/kg pretreated with normal saline or nontargeting scrambled siRNA (the levels of EBD: V_T 30 mL/kg with normal saline = 120.2 ± 4.9 ng/mg lung weight versus V_T 30 mL/kg with nontargeting scrambled siRNA = 127.1 ± 6.3 ng/mg lung weight, $P = 0.68$; wet-to-dry weight ratio: V_T 30 mL/kg with normal saline = 6.4 ± 0.3 versus V_T 30 mL/kg with nontargeting scrambled siRNA = 6.7 ± 0.2, $P = 0.27$).

Reduced VILI-associated inflammatory response by neutrophil elastase inhibitor

We next identified the cell types involved in this VILI model. The neutrophil counts and MPO assay revealed that neutrophils migrated into the injured lung sites in mice after mechanical ventilation at V_T30 when compared with nonventilated mice or mice receiving a V_T6 (Figure 3(a) and (b)). Meanwhile, the neutrophil elastase activity, MDA, which is an aldehydic secondary product of lipid peroxidation produced by neutrophils, and MIP-2 protein levels were elevated in response to V_T30 treatment (Figure 3(c) to (e)), indicating an increase of oxidative stress and upregulation of chemoattractants for neutrophils in this model. Significantly, neutrophil elastase inhibitor, SN50, or NRF siRNA ameliorated neutrophil migration, neutrophil elastase activity, MDA, and MIP-2 protein elevation (Figure 3). These data demonstrate that neutrophil elastase inhibitor, SN50, or NRF siRNA inhibitor abrogate neutrophil infiltration and inflammatory responses in high-tidal-volume-induced VILI.

Inhibition of mechanical ventilation-induced NF-κB/NRF pathway by neutrophil elastase inhibitor

Immunohistochemistry indicated that the airway epithelium stained positive for NRF or phospho-NF-κB after mechanical ventilation at V_T30, but not at V_T6 (Figures 4(a) and 4(b) and 5(a) and (b)). To further investigate the inter-relationship between NF-κB and NRF in this VILI model, we next used neutrophil elastase inhibitor, NRF siRNA or pharmacological NF-κB inhibition to identify the involvement of the NF-κB/NRF pathway in high-tidal-volume-induced VILI. Consistent with immunohistochemical findings, Western blot analyses revealed that NRF expression and phospho-NF-κB phosphorylation were increased in mice receiving mechanical ventilation at V_T30 and that NRF knockdown and inhibiting NF-κB with neutrophil elastase inhibitor or SN50 abolished the V_T30-induced NRF and phospho-NF-κB activation (Figures 4(c) and 5(c)). NRF mRNA upregulation in response to V_T30 was also prevented by NRF knockdown, pharmacologic inhibition with either neutrophil elastase inhibitor or SN50 (Figure 4d). Consistent with previous reports in airway inflammation,[5,8] our data indicate that NF-κB / NRF signaling is also required for the induction of VILI.

Reduced mechanical ventilation-induced epithelial apoptosis

Previous studies have revealed that NF-κB mediated epithelial apoptosis.[23,24] Since upregulation of NF-κB and NRF has been associated with stretch-induced pathway-driven lung inflammation, we performed an apoptosis assay (TUNEL stain and electron microscopy) to further determine the effects of neutrophil elastase inhibitor on ventilation-induced apoptosis of airway epithelial cells. The increases of V_T30-induced bronchial epithelial apoptosis of Clara cells were significantly lowered after pharmacological inhibition with neutrophil elastase inhibitor, SN50 or NRF siRNA (Figure 6). The epithelial apoptosis was further confirmed by electron micrographs that showed the characteristic nuclear condensation of bronchial epithelium in mice receiving high-tidal-volume mechanical ventilation (Figure 7a).

Reduced ultramicrostructural damage by neutrophil elastase inhibitor

Transmission electron microscopy was used to determine the effects of mechanical ventilation on the ultrastructures of bronchial epithelia (Figure 7a). The administration of V_T30 led to disruption of the airway ultramicrostructure: loss of microvilli, increases of secretary vesicles, and epithelial apoptosis characterized by nuclear condensation. Administration of neutrophil elastase inhibitor, SN50 or NRF siRNA, attenuated the development of airway injury. Furthermore, the PaO2/FiO2 ratio, an index of gas exchange, was significantly deteriorated with a V_T30 when compared with nonventilated mice or mice receiving a V_T6 (Figure 7b). Remarkably, the decreases in oxygenation with a V_T30 were significantly improved by the administration SN50 or NRF siRNA.

Discussion

VILI is characterized by diffuse inflammation, enhanced alveolar-capillary membrane permeability, accumulation of protein-rich pulmonary edema, ultimately leading to impaired gas exchange, termed biotrauma.[1,25] Ventilation-induced mediator release can result from stress failure of cell membranes, loss of epithelial and endothelial integrity, increased vascular shear stress, or mechanotransduction

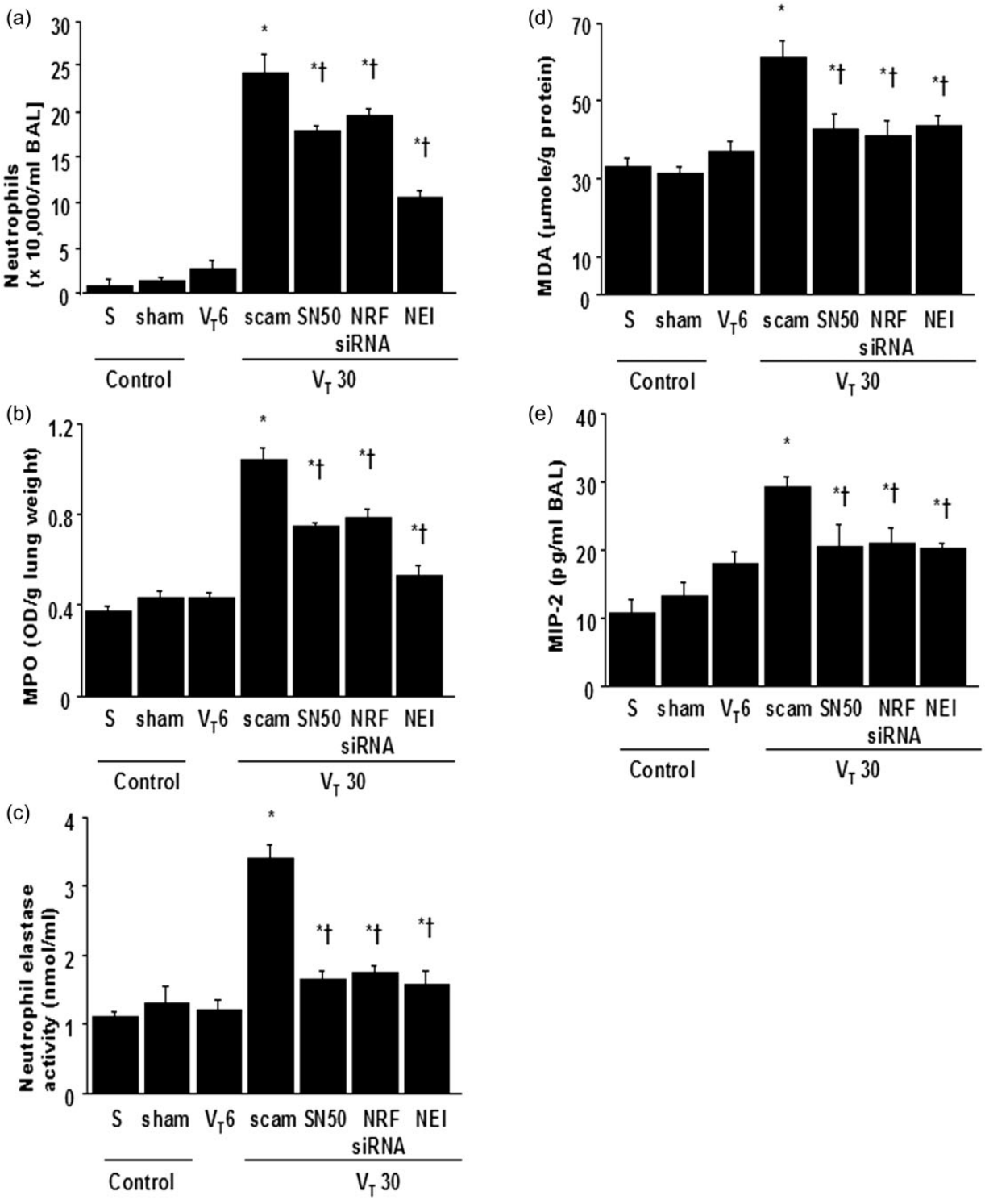

Figure 3 SN50, NRF siRNA, or neutrophil elastase inhibitor attenuated lung stretch-induced neutrophil infiltration and oxidant stress. The effects of administering SN50, NRF siRNA, or NEI on (a) neutrophil infiltration, (b) MPO activity, (c) neutrophil elastase activity, (d) MDA activity, and (e) MIP-2 secretion in BAL fluid in wild-type mice receiving mechanical ventilation at V_T6 or V_T30 are shown. Data shown here are the mean ± SD of five independent experiments. $^*P < 0.05$ vs. nonventilated control treated with normal saline or mice receiving a V_T 6 mL/kg, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. BAL: bronchoalveolar lavage fluid; MDA: malondialdehyde; MIP-2: macrophage inflammatory protein-2; MPO: myeloperoxidase

processes triggered by lung stretch. Previous *in vitro* and *in vivo* studies have shown that simply overdistending lung tissue, in the absence of any other stimuli, causes production of IL-8 and the activation of NF-κB.[10,12] Bartels and colleagues have revealed that peptide disruption of NRF and NF-κB interaction is crucial for the regulation of IL-8 promoter by IL-1 in HeLa cells.[14] Furthermore, experimental mouse models of VILI demonstrated that short duration

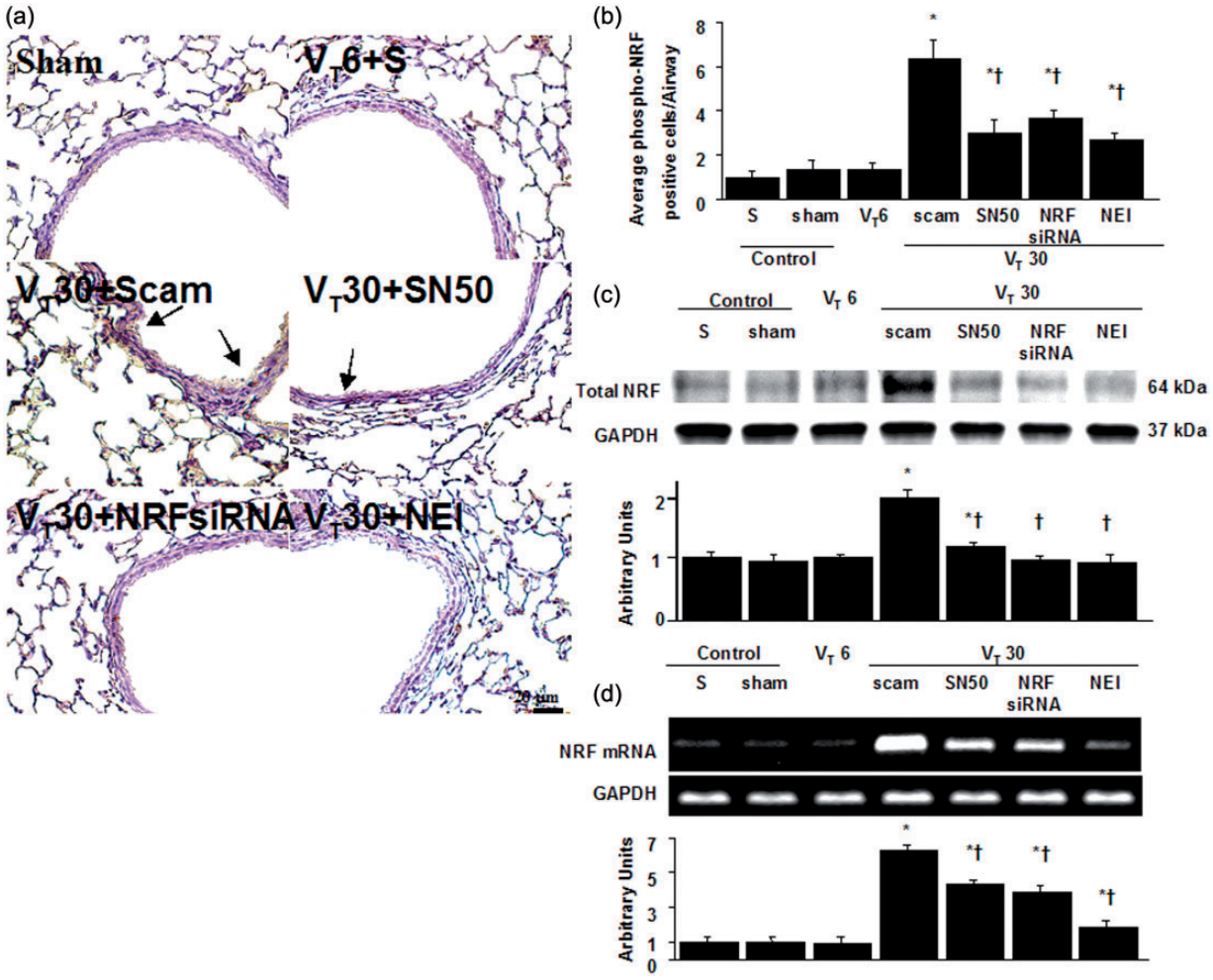

Figure 4 SN50, NRF siRNA, or neutrophil elastase inhibitor abrogated lung stretch-induced NRF and NRF mRNA expression. NRF expression from the lungs of V_T30-ventilated or V_T6-ventilated wild-type mice receiving SN-50, NRFsiRNA, or NEI as detected by either (a, b) immunohistochemistry (×400) and quantification or (c) Western blot analyses. The effects of administering of SN-50, NRFsiRNA, or NEI on the mRNA expression of (d) NRF are demonstrated. Arbitrary units are expressed as the ratio of NRF to GAPDH or NRF mRNA to GAPDH. A dark brown diaminobenzidine signal identified by arrows indicates positive staining for NRF in the lung epithelium or interstitial, whereas shades of bluish tan signify nonreactive cells. Data shown here are the mean ± SD of five independent experiments. *$P < 0.05$ vs. nonventilated control treated with normal saline or mice receiving a V_T 6 mL/kg, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. Scale bars represent 20 μm. GAPDH: glyceraldehyde 3-phosphate dehydrogenase. (A color version of this figure is available in the online journal.)

of mechanical ventilation, from 2 to 4 h, may induce expression of NF-κB, which was attenuated by the administration of hydrogen inhalation, hypercapnic acidosis, steroids, and toll-like receptor 4 mutant mice.[4,5,12,26] However, the relationship between mechanical ventilation, neutrophil infiltration, and NF-κB and NRF expression are still unclear and need to be evaluated in preclinical studies. ALI is characterized by impaired gas exchange of alveoli and airways, which includes terminal and respiratory bronchioles. Therefore, we explored high-tidal-volume ventilation-induced ALI in murine bronchial epithelium, besides the alveolar injury found in our (Figures 2(a), 6(a), and 7(a)) and others' studies in the mice.[7,10] We observed that neutrophil elastase inhibitor suppressed mechanical stretch-induced VILI, as observed by decreased histological evidence of lung injury, lung edema, microvascular permeability, neutrophil sequestration, neutrophil elastase, ROS, and MIP-2 production, but elevated PaO_2/F_IO_2 ratio in bronchial epithelium in response to these treatments.

Neutrophils are the main inflammatory cells involved in the process of ALI through the secretion of cytokines IL-1β, MIP-2, and TNF-α and play a vital role in the generation of neutrophil elastase and ROS.[6,7,27] ROS, including superoxide, hydrogen peroxide, and hydroxyl radical, may overwhelm the protection of antioxidants in ALI.[9,27] Neutrophil elastase has been involved in the increase of inflammation in alveolar epithelial cells through activation of NF-κB.[13,28] Additionally, neutrophil elastase inhibitor has effects on signal transduction cascades by reducing intracellular calcium ion influx and by controlling phospholipase C activity.[29] Importantly, previous clinical studies in critically ill patients showed that neutrophil elastase inhibitor improved the outcome of patients with ALI, including the length of stay in the intensive care unit, ventilator-free days,

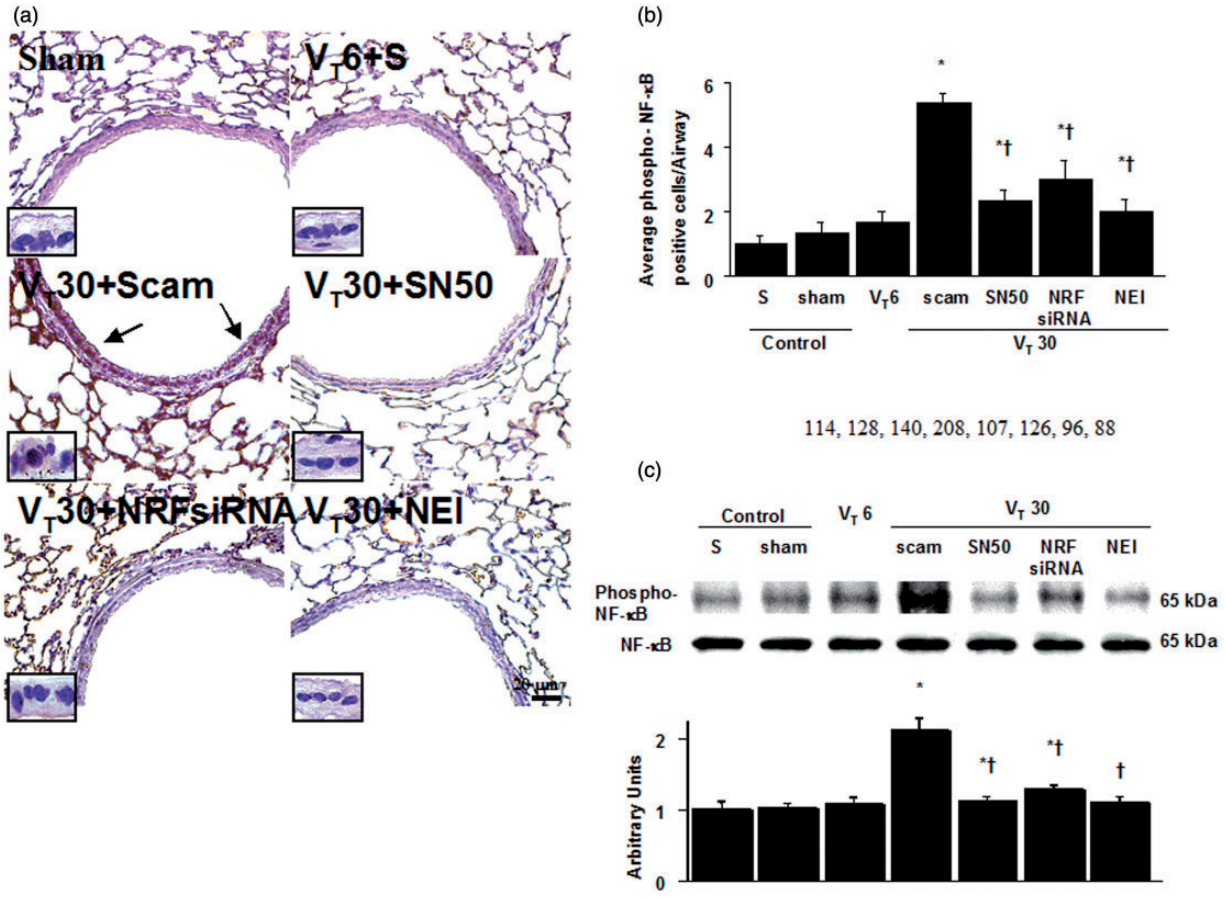

Figure 5 SN50, NRF siRNA, or neutrophil elastase inhibitor suppressed lung stretch-induced NF-κB phosphorylation. NF-κB phosphorylation from the lungs of V_T30-ventilated wild-type mice receiving SN-50, NRF siRNA, or NEI as detected by either (a, b) immunohistochemistry (×400) and quantification or (c) Western blot analyses. Arbitrary units are expressed as the ratio of phospho-NF-κB to NF-κB. An inset panel showed the translocation of NF-κB into the nucleus. A dark brown diaminobenzidine signal identified by arrows indicates positive staining for phospho-NF-κB in the lung epithelium or interstitial, whereas shades of bluish tan signify nonreactive cells. Data shown here are the mean ± SD of five independent experiments. *$P < 0.05$ vs. nonventilated control treated with normal saline or mice receiving a V_T 6 mL/kg, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. Scale bars represent 20 μm. (A color version of this figure is available in the online journal.)

oxygenation, multiple organ dysfunction score, and ventilator-weaning rate.[30–33] However, the STRIVE study, a clinical trial in ALI patients requiring mechanical ventilation, failed to demonstrate the efficacy of sivelestat.[34] The discrepancy might be due to differences in the severity of lung injury in the patients.[31–33] Neutrophil elastase inhibitor was found to be more effective in patients with $PaO_2/F_IO_2 \geq 140$ mmHg.[31] Our data indicate that high-tidal-volume ventilation induces the expression of NF-κB and NRF in bronchial epithelium, which can be suppressed by the administration of neutrophil elastase inhibitor, accompanied with reduced neutrophil infiltration, neutrophil elastase activity, and production of MDA and MIP-2, a functional homolog of human IL-8 belonging to the cysteine-amino-cysteine family of cytokines.[10]

NF-κB can modulate both proinflammatory and anti-inflammatory responses that lead to injury of lung epithelial cells after mechanical ventilation.[4,10,26] We previously observed *in vitro* A549 cellular stretch-induced NF-κB activation and IL-8 production.[10] The contribution of NF-κB was further validated by SN-50 treatment, which was a cell-membrane-permeable peptide that entered the cells by carrying a positively charged nuclear localization sequence and competed with NF-κB for nuclear translocation.[19] Recently, an *in vivo* study of VILI in rats also revealed that blockade of high stretch ventilation-induced NF-κB activation improved oxygenation, compliance, and histologic injury.[4] Furthermore, enhanced activation of NF-κB may aggravate the severity of ALI by production of MIP-2 and prolonging the presence of activated neutrophils in the lungs.[11,35] The effects of apoptosis vary with cell types and with the time after onset of ALI.[36] In early ARDS, alveolar and airway epithelial apoptosis are increased by the combined effects of increased concentration of Fas ligand, decreased concentration of surfactant protein A, and increased concentration of angiotensin II.[36] Although NF-κB is generally regarded as anti-apoptotic, NF-κB can also promote epithelial apoptosis through the nucleolar accumulation of RelA in response to cellular stress.[23,24] A previous study of leukocyte elastase-induced apoptosis of lung epithelial cells demonstrated that NF-κB activation could be pro-apoptotic and pharmacological inhibition of

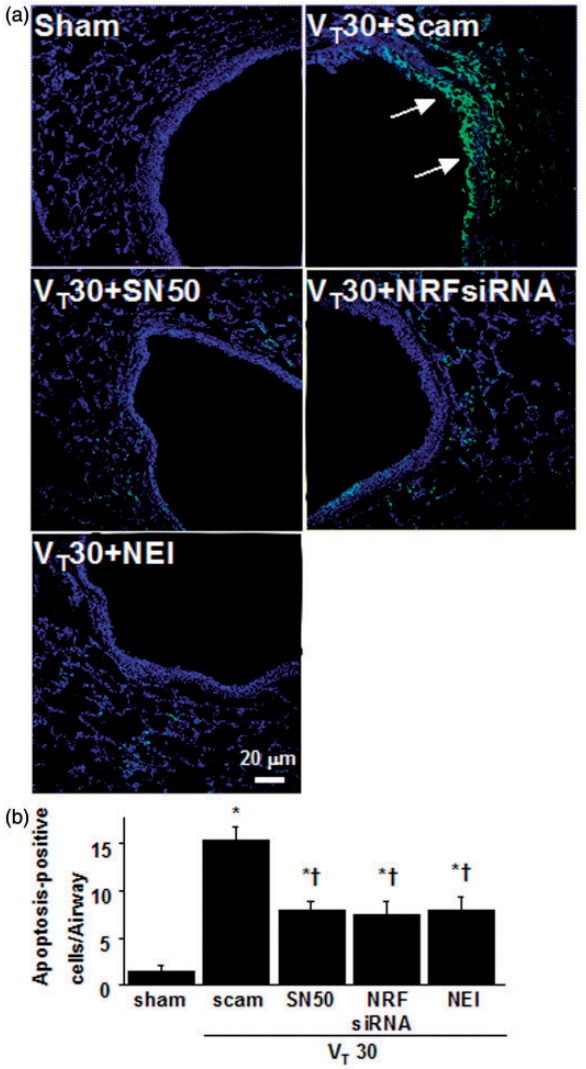

Figure 6 Effects of SN50, NRF siRNA, or neutrophil elastase inhibitor on lung stretch-induced bronchial epithelial apoptosis. The effects of administering SN50, NRF siRNA, or NEI on (a) TUNEL staining of paraffin lung sections (×400) and (b) quantification are shown. Data shown here are representative results of five independent experiments. Apoptotic cells are identified by arrows. A bright green signal indicates positive staining of apoptotic cells, and shades of dull green signify non-reactive cells. *$P < 0.05$ vs. nonventilated control treated with normal saline, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. Scale bars represent 20 μm. (A color version of this figure is available in the online journal.)

the leukocyte elastase and by the way, suppressing the NF-κB signaling was expected to reduce the lung epithelial apoptosis.[37] Recently, a mouse model of endotoxemia demonstrated that molecular hydrogen treatment attenuated lung inflammation and apoptosis through the decrease of NF-κB activity.[24] We found that mechanical ventilation-induced bronchial epithelial apoptosis through increases of neutrophil elastase and NF-κB/NRF signaling. Nevertheless, NF-κB may have dual roles and can be pro- or anti-inflammatory depending on the experimental model (positive or negative pressure, pressure or volume control mode, duration of ventilation, and *ex vivo* isolated lungs or *in vivo* lung model), cell type, and a subunit composition of the NF-κB complex.[4,5,10,24–26] Collectively, high stretch mechanical ventilation increased secretion of chemotactic factor MIP-2, neutrophil infiltration, generation of ROS, and release of neutrophil elastase. Moreover, neutrophil elastase may activate NF-κB by phosphorylating IκB and its transcriptional coactivators NRF, leading to further production of inflammatory cytokines and increased microvascular permeability and lung injury.[28,38–40] We further examined the roles of NRF expression in VILI modulated by NF-κB.

NRF is ubiquitously and constitutively expressed in all tested human tissues, including the lung.[8] By direct protein–protein interaction with NF-κB-dependent promoters, NRF represses the transcriptional process.[8] However, recent *in vitro* studies have revealed that NRF has a dual role in IL-8 transcription.[14,15,18,41] In the absence of stimulation, NRF is involved in transcriptional silencing, but, in the presence of IL-1β, it functions as a coactivator for full induction of the IL-8 promoter.[18] By binding to the NRE of the IL-8 promoter, NRF plays an additional role and acts as a positive component in the IL-8 gene expression. Furthermore, an *in vitro* study in IL-1-treated epithelial human cervical (HeLa) cells showed that a distinctive feature of IL-8 promoter is the cooperative interaction of NF-κB p65 with NRF to activate transcription in response to IL-1.[14] NRF and NF-κB p65 binding sites either apart or together are crucial for the activation of IL-8 promoter by IL-1 in HeLa cells.[18] The molecular basis for NRF to switch from a repressor to an activator is still elusive. This mechanism may depend on the cell types, location and timing of inducers, and the regulation and structure of the transcriptional complex.[18,42] Additionally, an *in vitro* study of C243 cells indicated that lung stretch-induced synergy of positive regulatory domains is sufficient to overcome the silencing effect of NRF.[15] Our study demonstrated that high-tidal-volume mechanical ventilation increased neutrophil influx and the production of neutrophil elastase and ROS, which may induce IL-8 gene transcription via NF-κB and NRF activation.[4,7,10,12,26,39–41] Inhibition of the production of ROS and neutrophil elastase by neutrophil elastase inhibitor attenuated VILI in mice. Using NF-κB inhibitor and NRF siRNA to mimic the effects of neutrophil elastase, we observed reduced morphological disruption, lung edema, microvascular permeability, production of MIP-2, and improvement of oxygenation. Moreover, upon administering neutrophil elastase inhibitor and SN50 together, we observed substantial improvement of lung injury, indicating a more pronounced inhibition of NF-κB activation. Collectively, our data validate the crucial role of NF-κB and NRF pathway in the pathogenesis of VILI, and blocking NF-κB and NRF signaling by neutrophil elastase inhibitor is at least partly responsible for the effect of sivelestat on VILI (Figure 8).

Our study has some limitations. Our previous study demonstrated that *in vitro* alveolar stretch-induced IL-8 production was dependent on c-Jun NH2-terminal kinases (JNK) and NF-κB-inducing kinase (NIK) activation, respectively.[10] Using 4 h of 20 mL/kg mechanical ventilation in mice, others have shown that sivelestat could reduce mechanical ventilation-induced inflammatory cytokines, JNK

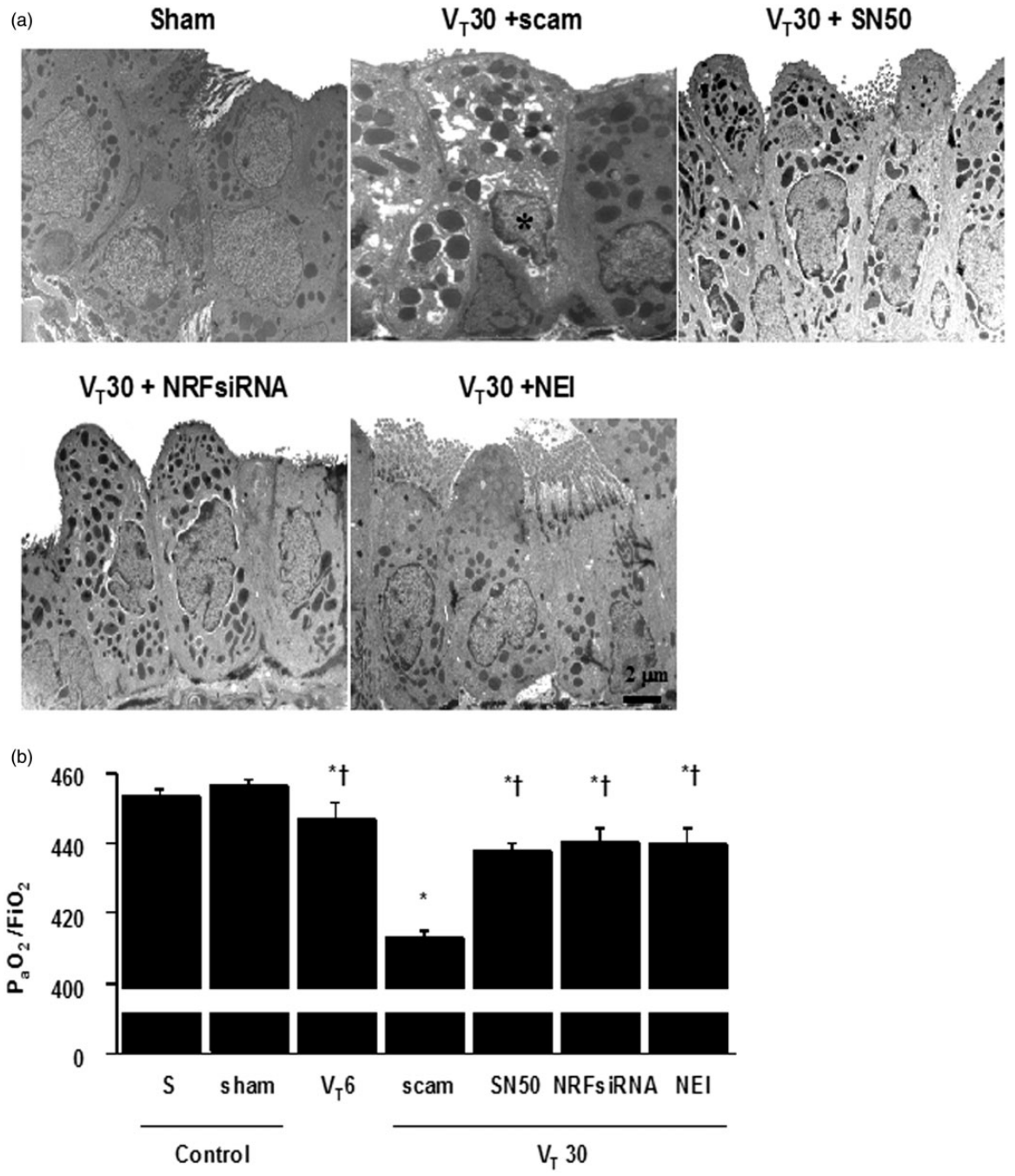

Figure 7 Attenuation of ultramicrostructure and oxygenation by SN50, NRF siRNA, or neutrophil elastase inhibitor. (a) A transmission electron microscopic image revealing the improvement of airway ultramicrostructure by SN50, NRF siRNA, or NEI treatment. (b) Improvement of oxygenation by SN50, NRF siRNA, or NEI in wild-type mice receiving high-tidal-volume or low-tidal-volume ventilation was shown. Data shown here are the mean ± SD of three independent experiments. Apoptotic cells are identified by asterisks. Highly condensed and fragmented heterochrmatin of bronchial epithelial cells indicates apoptosis. *$P < 0.05$ vs. nonventilated control treated with normal saline or mice receiving a V_T 6 mL/kg, †$P < 0.05$ vs. V_T30-ventilated mice treated with nontargeting scrambled siRNA. Scale bars represent 2 μm

activation, and alveolar apoptosis.[7] The role of mechanical ventilation-induced JNK signaling pathway was further confirmed by a *in vivo* study of mice treated with pharmacological inhibitor SP600125 and in JNK knockout mice.[43] In the present study, we observed that sivelestat attenuated mechanical ventilation-induced NF-κB, NRF, free radical production, and apoptosis in bronchial epithelium of mice. An *in vitro* study of the mapping of NRF binding domain in the NF-κB proteins shows that NRF significantly binds to the endogenous p65 subunit but not to the p50.[44] Unfortunately, mice homozygous for the $Rela^{tm1Bal}$ targeted mutation (Jackson laboratory stock number 002851) die at embryonic day 14 due to liver apoptosis and failure of hematopoiesis. Partial inhibition of lung injury through NF-κB inhibitor and NRF siRNA, compared with the controlled mice, suggested that NF-κB/NRF signaling was only one

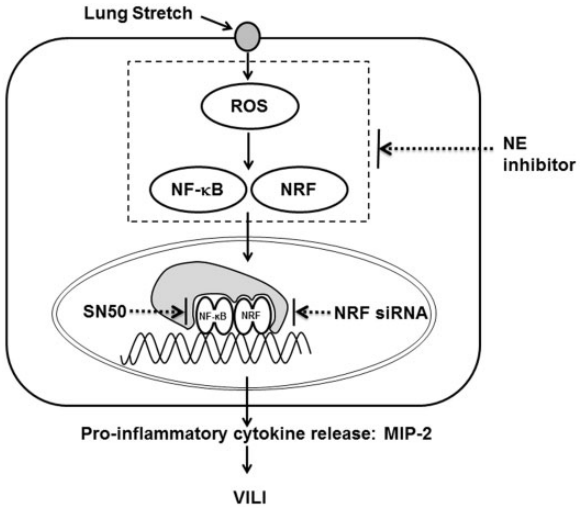

Figure 8 Schematic figure illustrating the signaling pathway activation with high-tidal-volume mechanical ventilation. Mechanical stretch-induced neutrophil infiltration, lung injury, and production of ROS, neutrophil elastase, and MIP-2 were attenuated with neutrophil elastase inhibitor through NF-κB/NRF pathway, demonstrating by using pharmacological inhibition of NF-κB and NRF activity by NF-κB inhibitor and NRF siRNA. The underlying mechanism predominantly involves the activation of NF-κB/NRF signaling. MIP-2: macrophage inflammatory protein-2; NE: neutrophil elastase; NF-κB: nuclear factor-κB; NRF: NF-κB repressing factor; ROS: reactive oxygen species; siRNA: short interfering RNA; VILI: ventilator-induced lung injury

of many pathways contributing to the protective effect of sivelestat. Further experiments are necessary to explore the effects of sivelestat on NF-κB binding to MIP-2a by chromatin immunoprecipitation.

Conclusion

We showed in a mouse model of ALI that high-tidal-volume mechanical ventilation-induced lung injury is associated with increased neutrophil influx and production of MIP-2, as well as overproduction of oxidative substances, which can be attenuated by neutrophil elastase inhibitor. The mechanisms that neutrophil elastase inhibitor suppressed these VILI characteristics involved inhibition of NF-κB and NRF pathway. Although lung-protective ventilation strategy is beneficial, the mortality of ARDS has remained substantially high.[45] Knowledge of the effect of mechanical forces on NF-κB and NRF allowed clarification of the pathophysiological mechanisms regulating acute exudative phase of ARDS, which may progress to irreversible pulmonary fibrosis and need the long-term use of ventilator. The neutrophil elastase inhibitor, which attenuates the activation of NF-κB and NRF pathway, may serve as a potential therapeutic reagent in the management of ALI/ARDS.

Author contributions: LF-L and YH-T conducted and designed the experiments. LF-L and YT-L collected and analyzed the data. LF-L and YY-L reviewed the references and wrote the manuscript. CH-C, KC-K, and MC-L reviewed the references.

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DECLARATION OF CONFLICTING INTEREST

The study sponsor has no role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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