Genetic and Pharmacologic Evidence Links Oxidative Stress to Ventilator-induced Lung Injury in Mice

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Rationale: Mechanical ventilation (MV) is an indispensable therapy for critically ill patients with acute lung injury and the adult respiratory distress syndrome. However, the mechanisms by which conventional MV induces lung injury remain unclear.

Objectives: We hypothesized that disruption of the gene encoding *Nrf2*, a transcription factor that regulates the induction of several antioxidant enzymes, enhances susceptibility to ventilator-induced lung injury (VILI) and that antioxidant supplementation attenuates this effect.

Methods: To test our hypothesis and to examine the relevance of oxidative stress in VILI, we assessed lung injury and inflammatory responses in *Nrf2*-deficient (*Nrf2*^{-/-}) mice and wild-type (*Nrf2*^{+/+}) mice after an acute (2-h) injurious model of MV with or without administration of antioxidant.

Measurements and Main Results: *Nrf2*^{-/-} mice displayed greater levels of lung alveolar and vascular permeability and inflammatory responses to MV as compared with *Nrf2*^{+/+} mice. *Nrf2* deficiency enhances the levels of several proinflammatory cytokines implicated in the pathogenesis of VILI. We found diminished levels of critical antioxidant enzymes and redox imbalance by MV in the lungs of *Nrf2*^{-/-} mice; however, antioxidant supplementation to *Nrf2*^{-/-} mice remarkably attenuated VILI. When subjected to a clinically relevant prolonged period of MV, *Nrf2*^{-/-} mice displayed greater levels of VILI than *Nrf2*^{+/+} mice. Expression profiling revealed lack of induction of several VILI genes, stress response and solute carrier proteins, and phosphatases in *Nrf2*^{-/-} mice.

Conclusions: Our data demonstrate for the first time a critical role for *Nrf2* in VILI, which confers protection against cellular responses induced by MV by modulating oxidative stress.

Keywords: acute lung injury; antioxidant enzymes; mechanical ventilation; *Nrf2*; inflammation

The adult respiratory distress syndrome (ARDS) and its less severe form, acute lung injury (ALI), have an average incidence of 150,000 cases per year and carry substantial mortality ranging from 30 to 40% (1). Although mortality has improved over the past few years, patients with ALI/ARDS are faced with an increased risk of death, and survivors are left with significant decrements in physical function. Mechanical ventilation (MV) is

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanisms by which mechanical ventilation contributes to lung injury are not well understood.

What This Study Adds to this Field

This study suggests that the pathogenesis of ventilator-associated lung injury involves redox imbalance in the lungs and that the transcription factor *Nrf2* has a key protective role by reducing oxidative stress.

the only known effective and cornerstone therapy used to support patients with ALI or ARDS. A lung-protective strategy of MV at low V_T (6 ml/kg) showed a beneficial effect in reducing mortality in patients with ALI/ARDS compared with conventional high V_T (HVT) (12 ml/kg), suggesting that mechanical and shear forces generated by HVT can enhance or perpetuate ventilator-induced lung injury (VILI) (2). Conventional MV at HVT causes excessive alveolar distention, resulting in lung injury and increased pulmonary vascular permeability and in an increase in the production of proinflammatory mediators (3). *In vitro* studies, using cyclic stretch that mimics the physical forces of MV, demonstrated that endothelial and epithelial cell deformation and physical disruption of plasma membrane integrity lead to alteration in the structure and function of these essential components of the alveolar-capillary membrane, along with activation of proinflammatory and prooxidant pathways (4–7). These events may serve to initiate and/or potentiate an inflammatory response, leading to a vicious cycle of inflammation locally or systemically, ultimately resulting in multisystem organ dysfunction and death (8). Cytokines and chemokines are potential effector molecules that modulate and regulate VILI (8). The accumulation of neutrophils in the lung tissue can also lead to enhanced levels of inflammatory cytokines, which play fundamental roles in the development of lung pathogenesis, including VILI (9, 10). Recent expression profiling using injurious and non-injurious MV in the presence and absence of inflammatory stimuli revealed the involvement of a complex network of genes encoding for matrix remodeling proteins, proinflammatory cytokines, and various transcription factors in VILI (11–15). The exact molecular mechanisms controlling the expression levels of these genes in response to MV and their contribution to the development or susceptibility to VILI are unclear. Thus, further deciphering the mechanisms by which conventional MV exacerbates lung injury and inflammation is of considerable clinical significance, not only in alleviating the side effects of mechanical forces but also for the development of new therapeutic strategies.

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Production of high levels of reactive oxygen species (ROS) and reactive nitrogen species is a condition that generally leads to oxidative stress. The induction of cytoprotective antioxidant enzymes in response to injurious insults or stressful stimuli is critical for cellular detoxification of ROS and reactive nitrogen species and in the maintenance of homeostasis of cellular redox. Emerging experimental evidence from animal models suggests that oxidative stress contributes to and/or exacerbates various clinical syndromes, such as ARDS, sepsis, shock, major surgery, and ALI (16–18). However, the exact relevance of redox imbalance to the onset of MV-induced lung injury *in vivo* remains unclear. Recent studies have shown that cyclic stretch associated with conventional MV at HV_T generates ROS and redox imbalance in lung epithelial and endothelial cells *in vitro* (19–21). Consistent with the *in vitro* studies are a decrease in antioxidant activity in the lungs and enhanced levels of oxidative stress plasma markers, such as thiobarbituric reactive substances and malondialdehyde, that are observed in response to MV *in vivo* (22, 23). Based on these observations, we hypothesized that redox imbalance caused by conventional MV might initiate and/or amplify lung cellular injury and inflammatory responses, which promote and/or perpetuate the pathogenesis of VILI.

Nrf2 is a transcription factor and regulates cellular redox status via the antioxidant response element (24). Genetic disruption of Nrf2 enhances susceptibility to various experimental murine models of lung diseases produced by prooxidants (25–28), mainly due to decreased levels of the basal and inducible expression of several critical antioxidant enzymes. Some of the critical enzymes include glutamate–cysteine ligase catalytic subunit (Gclc), glutamate cysteine ligase modifier subunit (Gclm), glutathione peroxidase 2 (Gpx2), and hemoxygenase 1 (24). To test our hypothesis and to determine the contribution of oxidative stress to the pathogenesis of VILI *in vivo*, we compared lung injury and inflammatory responses in mice with disruption of the *Nrf2* gene (*Nrf2*^{−/−}) versus wild-type mice in response to injurious MV. Here we demonstrate that disruption of *Nrf2* enhances susceptibility to MV-induced lung injury and inflammation and elevates levels of proinflammatory cytokine expression. We found diminished levels of antioxidant enzyme expression and redox imbalance in the lungs of *Nrf2*^{−/−} mice subjected to MV as compared with wild-type animals. Finally, antioxidant supplementation attenuated MV-induced lung injury and inflammation in both genotypes, supporting a role for oxidative stress in the pathogenesis of VILI. Results from this work have previously been published in abstract form (29).

METHODS

Reagents

The *Nrf2* wild-type (*Nrf2*^{+/+}) and *Nrf2*-deficient (*Nrf2*^{−/−}) CD-1/ICR strains of mice were generated as described (40). Mice were fed with normal diet and water *ad libitum* and were housed under controlled conditions (25 ± 2°C; 12-h light:dark periods). All experimental animal protocols were performed in accordance with guidelines approved by the animal care use committee at the Johns Hopkins University Bloomberg School of Public Health.

MV

Exposure to MV was performed as previously described (30–32). Briefly, 6- to 8-week-old male mice (25–30 g) were used for these studies unless otherwise indicated. Mice were anesthetized with pentobarbital (intraperitoneally, 80 mg/kg body weight), and additional anesthetic was supplemented regularly at approximately 20 mg/kg per hour for maintaining the plane of anesthesia during the experiment period. Rectal temperature was continuously monitored and main-

tained at 37 ± 1°C. A neck midline incision was performed for exposure of the trachea to facilitate endotracheal intubation with a 20-gauge, 1-inch-long catheter (Johnson and Johnson, New Brunswick, NJ). The animals were subjected to MV (Model Inspira asv 55-7058; Harvard Apparatus, Boston, MA) with low V_T (LV_T) and HV_T at 12 ml/kg and 30 ml/kg, respectively, for 2 hours. The animals subjected to spontaneous ventilation (SpV) or MV with LV_T (12 ml/kg) for 4 hours were supplied with phosphate-buffered saline (PBS) (1 µl/mg body weight) at 30-minute intervals.

The respiratory rate was set at 80 to 140 breaths per minute depending on V_T. The adequacy of MV settings on gas exchange was confirmed in preliminary experiments in which arterial blood gases obtained via catheterization of a femoral artery were analyzed by an automated blood gas analyzer (model 348 Blood Gas Analyzer; Chiron Diagnostics; Norwood, MA) as previously described (31, 32). Arterial blood gas analysis revealed stable levels of arterial oxygen (Pa_O₂ of 55–90 mm Hg) and carbon dioxide (Pa_{CO}₂ of 30–50 mm Hg) (Table 1). Sham-operated, anesthetized, and SpV mice were used as control groups. Animals were given a lethal dose of the anesthetic agent before the lungs were harvested.

Assessment of Lung Injury and Inflammation in Bronchoalveolar Lavage Fluid

Lung injury was assessed by alveolar and microvascular permeability. Lung inflammation was evaluated by differential cell counts in bronchoalveolar lavage fluid (BALF) as previously described (25). Lungs were instilled with 1.5 ml of sterile PBS, and cells in the BALF were counted using a hemocytometer. Differential cell counts were assessed with Diff-Quik stain set (Dade Behring, Inc., Newark, DE). The remainder of the BALF was centrifuged, and the supernatant was stored at −80°C. BALF protein concentration was measured by BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). Lung vascular permeability was determined using Evans blue injections as previously detailed (30–32). Extravasated Evans blue concentration in lung homogenates was calculated against a standard curve and expressed as micrograms of Evans blue per milligram of lung tissue as previously described (30–32).

Mouse Albumin ELISA

The albumin concentration in the BALF was determined by ELISA (E90-134; Bethyl Laboratories, Montgomery, TX) using a protocol recommended by the manufacturer.

Measurement of Reduced and Oxidized Glutathione Levels

A Bioxytech GSH/GSSG-412 kit (Oxis Health Products, Portland, OR) was used to measure reduced and oxidized glutathione in the lung homogenates as per the manufacturer's instructions and as previously described (21).

Supplementation of Antioxidant

To assess the effects of antioxidants on MV-induced pulmonary and vascular permeability and inflammation, mice received in some experiments a single intraperitoneal dose of the antioxidant *N*-acetyl-L-cysteine (NAC) (100 mg/kg body weight) or a similar volume of vehicle (PBS) 60 minutes before exposure to MV.

Immunohistochemical Analysis

At the end of 2 hours of MV, lungs were inflated to 25 cm of water pressure with 0.5% of low-melting agarose with 10% buffered formalin. Lungs were harvested and stored in 10% formalin for 24 hours at 4°C before embedding in paraffin. Sections (5-µm) were cut and stained with hematoxylin and eosin. For identification of neutrophils, deparaffinized tissue sections were incubated with rat antimouse neutrophil monoclonal antibody (Serotec, Raleigh, NC) followed by biotinylated antirat secondary antibody and horseradish peroxidase–streptavidin complex. Lung sections were acquired with a Nikon E 800 microscope (Nikon, Melville, NY). The number of neutrophils in the lung sections (n = 5 per group and 15 fields per lung section per animal) were counted manually by an investigator blinded to the various groups using a grid system. The number of neutrophils was normalized per field for each animal and expressed as mean ± SE as previously described (30).

TABLE 1. DIFFERENTIAL EXPRESSION PROFILING OF GENES ALTERED BY MECHANICAL VENTILATION IN THE LUNGS OF *Nrf2*-DEFICIENT (*Nrf2*^{-/-}) AND WILD-TYPE (*Nrf2*^{+/+}) MICE

Gene Symbol	Gene Title	MV vs. SpV <i>Nrf2</i> ^{+/+}	MV vs. SpV <i>Nrf2</i> ^{-/-}
Tissue Remodeling and Coagulation Proteins			
Areg	Amphiregulin	17.27	5.54
Adamts1	A disintegrin-like and metalloprotease	1.69	
Btg2	B-cell translocation gene 2, antiproliferative	1.68	
F3	Coagulation factor III	4.26	2.3
Hbegf	Heparin-binding EGF-like growth factor	2.75	
Plaur	Urokinase plasminogen activator receptor	2.48	
Pdgfb	Platelet-derived growth factor, B polypeptide	-1.52	
Ptgs2	Prostaglandin-endoperoxide synthase 2	6.23	5.98
Serpine1	Serine (or cysteine) proteinase inhibitor, clade E, member 1	3.71	
Serpina3n	Serine (or cysteine) proteinase inhibitor, clade A, member 3N	1.59	
Thbs1	Thrombospondin 1	5.24	
Timp3	Tissue inhibitor of metalloproteinase 3	1.83	
Regulators of Inflammation			
Il6	Interleukin 6	7.78	
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	2.66	
Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19	-1.69	
Traf1	Tnf receptor-associated factor 1	-1.83	
Cxcl1	Chemokine (C-X-C motif) ligand 1	2.55	
Il1b	Interleukin 1 beta		-2.20
Cxcl2	Chemokine (C-X-C motif) ligand 2		-2.51
Ifi203	Interferon activated gene 203		14.03
Ildf1	Interferon-related developmental regulator 1	2.03	
Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	-3.07	
Ilgp1	Interferon-inducible GTPase 1	-1.54	
Socs3	Suppressor of cytokine signaling 3	5.24	
Socs4	Suppressor of cytokine signaling 4		2.51
Redox and Stress Regulators			
Txnrd1	Thioredoxin reductase 1	1.91	
Gclc	Glutamate-cysteine ligase, catalytic subunit	1.82	
Cyr61	Cysteine rich protein 61	4.26	
Ncf2	Neutrophil cytosolic factor 2		-1.85
Ncf4	Neutrophil cytosolic factor 4		-1.79
Nox4	NADPH oxidase 4		-2.10
S100a9	S100 calcium-binding protein A9 (calgranulin B)		-5.43
S100a8	S100 calcium-binding protein A8 (calgranulin A)		-5.28
Carhsp1	Calcium-regulated heat stable protein 1		-1.77
Dnaja1	DnaJ (Hsp40) homolog, subfamily A, member 1		-2.03
Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1		-2.64
Hspa1a	Heat shock 70-kDa protein 1A		-4.99
Hspa1b	Heat shock 70-kDa protein 1B		-7.89
Hspa8	Heat shock 70-kD protein 8		-1.67
Hsp105	Heat shock protein 105	-1.83	
Hspa12b	Heat shock protein 12B	-2.69	
Mt1	Metallothionein 1	4.06	
Mt2	Metallothionein 2	4.96	
Transcription Factors			
Atf3	Activating transcription factor 3	7.01	1.95
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	1.61	
Egr1	Early growth response 1	5.21	-2.51
Egr2	Early growth response 2	3.76	
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	2.48	
Fos	FBJ osteosarcoma oncogene	4.92	
Fosb	FBJ osteosarcoma oncogene B	5.06	
Klf6	Kruppel-like factor 6	2.77	
Klf7	Kruppel-like factor 7	1.51	
Maff	v-maf protein F	5.24	
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	3.84	
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	3.16	
Srf	Serum response factor	2.27	
Smad1	MAD homolog 1 (Drosophila)	1.51	
Trib1	Tribbles homolog 1 (Drosophila)	6.92	
Ppargc1a	PPAR gamma coactivator 1 alpha	2.62	2.45
Sox18	SRY-box containing gene 18	-3.34	
Sox7	SRY-box containing gene 7	-1.91	
Stat5b	Signal transducer and activator of transcription 5B	-1.51	

(Continued)

TABLE 1. (CONTINUED)

Gene Symbol	Gene Title	MV vs. SpV <i>Nrf2</i> ^{+/+}	MV vs. SpV <i>Nrf2</i> ^{-/-}
Kinases and Phosphatases			
Dusp1	Dual-specificity phosphatase 1	2.08	
Dusp8	Dual-specificity phosphatase 8	2.25	
Dusp16	Dual-specificity phosphatase 16	1.73	
Ptp4a1	Protein tyrosine phosphatase 4a1	2.99	2.01
Ptpn2	Protein tyrosine phosphatase, nonreceptor type 2	1.61	
Ppp2r2a	Protein phosphatase 2 regulatory subunit B, alpha isoform		2.11
Bmx	BMX nonreceptor tyrosine kinase	1.85	
Ripk4	Receptor-interacting serine-threonine kinase 4	1.78	
Hk2	Hexokinase 2	1.78	
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	2.73	
Tob1	Transducer of ErbB-2.1		1.72
Tob2	Transducer of ERBB2, 2		1.66
Transporter Proteins			
Slc2a1	Solute carrier family 2 (glucose transporter), member 1	1.57	
Slca41	Solute carrier family 4 (anion exchanger) member 1	-3.12	-2.71
Slc7a5	Solute carrier family 7 (cationic amino acid transporter), member 5	1.87	
Slc20a1	Solute carrier family 20 (sodium/hydrogen exchanger), member 1	2.06	
Slc23a2	Solute carrier family 23 (nucleobase transporter), member 2	1.57	
Slc25a4	Solute carrier family 25 (mitochondrial carrier), member 4		1.85
Slc38a2	Solute carrier family 38 (neutral amino acids transporter), member 2	1.67	1.51
Slc38a4	Solute carrier family 38 (neutral amino acids transporter), member 4	2.57	

Definition of abbreviations: MV = mechanical ventilation; SpV = spontaneous ventilation.

Mice ($n = 3$ per group) were subjected to SpV or MV at high V_T for 2 hours. RNA was isolated, and microarray analysis was performed as detailed in METHODS. Data are represented as mean fold change obtained from comparing samples of MV with the SpV control group of the same genotype. All the represented fold-change values are significant ($P < 0.05$). VALI/VILI genes identified from the previous studies are listed in bold.

Western Blot Analysis

Right lung tissues were homogenized, and the protein concentration of cytosolic fraction of each sample was estimated using BCA reagent (Pierce) and stored at -70°C . Equal amounts of protein were separated and immunoblotted with specific antibodies as indicated. The blots were visualized with the ECL Western blot detection system. Autoradiogram signals were quantified by densitometry scanning (Molecular Dynamics, Sunnyvale, CA), and values were normalized to those of house-keeping gene β -actin and Hsp70.

Gene Expression Profiling

Nrf2^{+/+} and *Nrf2*^{-/-} mice were subjected to MV (HV_T) or SpV for 2 hours. Lungs were immediately removed and processed for total RNA isolation using TRIzol reagent (Life Technologies, Grand Island, NY). The isolated RNA was applied to Murine Genome UoA GeneChip arrays (Affymetrix, Santa Clara, CA), which contain probes for detecting approximately 14,500 well characterized genes and 4,371 expressed sequence tags according to standard microarray protocol. Scanned output files were analyzed by using Affymetrix GeneChip Operating Software and were independently normalized to an average intensity of 500. Further analyses were done by performing nine pairwise comparisons for each group (*Nrf2*^{+/+} SpV [$n = 3$] vs. *Nrf2*^{+/+} MV [$n = 3$]; *Nrf2*^{-/-} SpV [$n = 3$], *Nrf2*^{-/-} MV [$n = 3$], and *Nrf2*^{+/+} SpV [$n = 3$] vs. *Nrf2*^{-/-} SpV [$n = 3$]). To minimize the number of false positives, only altered genes that showed a change of ≥ 1.5 -fold and appeared in at least six of the nine comparisons were selected as described previously (27, 28). In addition, the Mann-Whitney pairwise comparison test was performed to rank the results by concordance as an indication of the significance ($P < 0.05$) of each identified change in gene expression.

Real-Time Polymerase Chain Reaction

The mRNA levels of genes encoding mouse *Gpx2*, *Gclc*, *Gclm*, IL-6, amphiregulin, early growth response gene (*Egr*)-1, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and actin-beta (β -actin) in the lungs of mice ($n = 3$ –4 per group) were quantified in triplicate by TaqMan gene expression assays according to the supplier's recommendations (Applied Biosystems, Foster City, CA) as previously described (21). The absolute values for each gene were normalized to that of *Gapdh*/ β -actin.

Measurement of Cytokines in the Lung Tissue

Lung tissues were homogenized at 4°C in cell lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1% Nonidet-P40, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_5 , 1 mM PMSF) and 1X protease inhibitor cocktail (Cat. #P8340; Sigma Chemical Co., St. Louis, MO) using a Brinkman polytron. The homogenates were centrifuged at 12,000 rpm for 15 minutes at 4°C . The supernatants were collected, and protein concentration was determined by BCA method (Pierce Biochemicals, Rockford, IL). Up to 100 μg of total protein was used for the cytokine multiplex bead-based immunoassay kit as per the manufacturer's recommendations (Bio-Rad Laboratories, Inc., Hercules, CA). Each supernatant sample was incubated with a mixture of all microbead types for 90 minutes at room temperature. Samples were then washed, incubated with a mixture of secondary biotinylated antibodies directed against each target for 30 minutes at room temperature, washed again, and incubated with a streptavidin-coupled phycoerythrin reporter system for 10 minutes at room temperature. After a final wash, the samples were suspended in buffer and subjected to flow-cytometric analysis. The data were analyzed using Bio-Plex Manager 3.0 software (Bio-Rad Laboratories, Inc.). For measurement of macrophage inflammatory protein (MIP)-2, extracts of lung tissue prepared as described previously were analyzed with a commercially available ELISA kit for mouse MIP-2 according to the manufacturer's suggestions (R&D Systems, Minneapolis, MN).

Statistical Analysis

All data involving animal experimentation were collected in a double-blind fashion. Data were expressed as the mean $\pm$ SEM ($n = 3$ –5 for each condition). Analysis of variance was used to compare means of multiple groups. For paired data, Student's *t* test was used. Significance in all cases was defined as $P \leq 0.05$.

RESULTS

Nrf2 Deficiency Enhances MV-induced Lung Permeability and Neutrophil Infiltration

One of the objectives of this study was to determine whether oxidative stress associated with conventional MV alone contrib-

uted to and/or exacerbated VILI and, if so, whether antioxidant supplementation could rescue these phenotypic effects. To examine the role of oxidative stress modifier Nrf2 in VILI, wild-type (*Nrf2*^{+/+}) and *Nrf2*^{-/-} mice were subjected to MV at LV_T (12 ml/kg) or HV_T (30 ml/kg) for 2 hours. Sham-operated, anesthetized, and SpV *Nrf2*^{+/+} and *Nrf2*^{-/-} mice were used as respective control groups. We found no trend differences in peak airway pressures between the wild-type and *Nrf2*^{-/-} mice subjected to LV_T or HV_T (data not shown). There was a small increase in airway pressure (average of 1 and 3 cm H₂O for LV_T and HV_T, respectively) over time (at 60 and 120 min) compared with baseline (time 0) value in both genotypes. Arterial blood gas analysis revealed no significant differences in the levels of arterial oxygen (Pa_O₂ of 55–90 mm Hg) and carbon dioxide (Pa_{CO}₂ of 30–50 mm Hg) (data not shown). We have previously reported no significant differences in alveolar diameter, alveolar cell proliferation, ultrastructural organization, and total lung capacity between *Nrf2*^{+/+} mice and *Nrf2*^{-/-} mice (27).

The effects MV on lung alveolar permeability and neutrophil accumulation in BALF were compared in both genotypes subjected to LV_T and HV_T (Figure 1). There was no difference in BALF protein levels in *Nrf2*^{+/+} and *Nrf2*^{-/-} mice in response to LV_T as compared with sham-operated (SpV) control groups (Figure 1A). However, we found statistically significant effects of MV for total protein accumulation in the BALF of wild-type and *Nrf2*^{-/-} mice as compared with the respective SpV control groups ($P < 0.05$). We also measured the concentration of albumin, as a measure of vascular leak, in the BALF. We did not find significant differences in serum albumin concentration between the two groups (data not shown).

This assay revealed significant differences in the amount of albumin content in BALF between the two genotypes in response to HV_T but not to LV_T. Compared with genotype-matched SpV controls, HV_T caused a statistically significant increase in mean albumin concentration in *Nrf2*^{+/+} mice (25.7 ± 3 vs. 20.4 ± 2.0 μ g/mg in HV_T compared with the SpV group; $P < 0.04$) and *Nrf2*^{-/-} mice (32.4 ± 1.29 vs. 21.04 ± 2.0 μ g/mg in HV_T compared with the SpV group; $P < 0.001$). However, the amount of albumin found in the BALF of *Nrf2*^{-/-} mice was significantly higher than that in *Nrf2*^{+/+} mice subjected to HV_T (32.4 ± 1.29 vs. 25.7 ± 3 , respectively; $P < 0.001$) (Figure 1B).

Differential cell count analysis revealed a significantly higher percentage of neutrophils in the BALF of *Nrf2*^{-/-} mice (21%) than wild-type animals (11%) (Figure 1C) after HV_T. We also found significantly higher levels of neutrophils in *Nrf2*^{-/-} mice subjected to MV at LV_T (8%) as compared with mice subjected to SpV (3%). No appreciable change in the levels of neutrophils was found in wild-type animals in response to LV_T (2%) as compared with SpV (1%). These results demonstrate that disruption of Nrf2 enhances susceptibility to MV-induced lung injury and inflammation.

Disruption of Nrf2 Augments MV-induced Lung Vascular Leak and Neutrophil Infiltration

Lung vascular leak and leukocyte infiltration is one of the hallmarks of ARDS. To assess the effects of MV on lung leukocyte infiltration, mice were subjected to HV_T and SpV, after which lungs were fixed with formalin and embedded in paraffin. Tissue sections were stained with antineutrophil antibody (Figure 2A), and the number of neutrophils present in the alveolar septa was quantified (Figure 2B) as detailed in METHODS. Immunohistochemical analysis showed no statistically significant difference in the number of neutrophils in both genotypes subjected to SpV (Figures 2A and 2B). Although there was a statistically significant increased number of neutrophils present in the lungs of *Nrf2*^{+/+} mice subjected to HV_T (10.4 ± 1.9 cells) compared with the SpV control group (6.57 ± 0.82 cells), *Nrf2*^{-/-} mice subjected to HV_T had greater number of neutrophils compared with the SpV control group (16.7 ± 2.6 vs. 8.2 ± 0.7 cells, respectively; $P < 0.01$). There was a greater number of neutrophils ($P < 0.04$) in the lungs of *Nrf2*^{-/-} mice than in *Nrf2*^{+/+} mice in response to MV.

To assess whether Nrf2 deficiency enhances susceptibility to vascular leak in response to MV, a separate group of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice was subjected to HV_T, and the results were compared with those of respective SpV control groups. We assessed lung vascular permeability using the Evans blue dye technique as previously described (30–32). Exposure to MV at LV_T caused no significant increase in extravasation of Evans blue in both genotypes compared with the respective SpV control groups (results not shown). However, exposure to HV_T caused significant

Figure 1. Effect of mechanical ventilation (MV) on lung injury and inflammatory responses in *Nrf2*^{-/-} mice. (A) Mice ($n = 3$ –5 per group) were subjected to MV at low V_T (LV_T, 12 ml/kg) and high V_T (HV_T, 30 ml/kg) for 2 hours and allowed to recover for 1 hour before bronchoalveolar lavage fluid (BALF) was obtained and protein was quantified as detailed in METHODS. In parallel, control mice were sham-operated, anesthetized, and spontaneously ventilated (SpV) under identical conditions of MV exposure. (A) The effect of LV_T and HV_T on BAL protein leakage in *Nrf2*^{+/+} and *Nrf2*^{-/-} mice. * $P \leq 0.05$ versus SpV control of the same genotype. (B) Albumin concentration in the BALF of various experimental groups (as in A) was determined using ELISA. * $P \leq 0.05$ versus SpV or LV_T groups of the same genotype. # $P \leq 0.05$, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to HV_T. (C) The number of neutrophils in the BALF collected from the *Nrf2*^{-/-} mice and *Nrf2*^{+/+} mice was counted and expressed as a percentage (top) of total cells (bottom). Values represent means $\pm$ SEM ($n = 3$ –5). * $P \leq 0.05$ versus SpV control of the same genotype; # $P \leq 0.05$, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to HV_T.

Figure 2. Effect of mechanical ventilation (MV) on pulmonary neutrophil infiltration and vascular protein leakage in *Nrf2*^{-/-} mice. (A) Assessment of neutrophil infiltration in lung parenchyma of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice subjected to high V_T (HV_T). Mice (n = 7–8 mice/group) were subjected to MV at HV_T for 2 hours, and then lungs were immediately fixed with 0.2% of low-melting agarose with 10% buffered formalin at 25 cm of water pressure. Immunostaining analysis of parenchymal neutrophils in the tissue sections was assessed using specific antineutrophil antibody. Original magnification: ×10. (B) Neutrophils in the alveolar space were quantified using digital images of tissue sections stained with antineutrophil antibody. Data represent means ± SEM of an average of at least 15 high-power fields for each exposure group. **P* ≤ 0.04, HV_T versus spontaneous ventilation (SpV) of *Nrf2*^{+/+} mice. ***P* ≤ 0.01, HV_T versus SpV of *Nrf2*^{-/-} mice. #*P* ≤ 0.04, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to HV_T. (C) Vascular leakage was assessed by the extravasation of Evans blue into lung parenchyma. Mice (n = 6 per group) were injected with Evans blue into the jugular vein 30 minutes before termination of MV at HV_T for 2 hours. Mice in the SpV group also received Evans blue. Lungs were immediately frozen, and Evans blue in the lung tissues of all experimental groups was quantified and expressed as μg/mg lung tissue. Values represent n = 6 per experimental condition. **P* ≤ 0.02, HV_T versus SpV of *Nrf2*^{+/+} mice. ***P* ≤ 0.001, HV_T versus SpV of *Nrf2*^{-/-} mice.

accumulation of Evans blue in the lungs of *Nrf2*^{+/+} (0.366 ± 0.074 vs. 0.172 ± 0.03 μg/mg in HV_T compared with the SpV group, respectively; *P* < 0.02) and *Nrf2*^{-/-} mice (0.428 ± 0.056 vs. 0.191 ± 0.06 μg/mg in HV_T compared with the SpV group, respectively; *P* < 0.001) (Figure 2C). Although there was no significant difference between these genotypes exposed to HV_T, there was a trend toward greater levels of Evans blue accumulation in the lungs of *Nrf2*^{-/-} mice as compared with *Nrf2*^{+/+} mice. Taken together, these results demonstrate that deficiency of the oxidative stress modifier Nrf2 may greatly enhance susceptibility to VILI.

Nrf2 Deficiency Augments MV-induced Lung Proinflammatory Cytokines Expression

Cytokines and chemokines are potential effector molecules that modulate and regulate VILI (8). We therefore assessed the levels of various cytokines present in the lungs of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice subjected to HV_T using multiplex cytokine assays as detailed in METHODS. Sham-operated (SpV) respective control groups were used for basal level expression. Cytokine assays revealed high levels of IL-6, KC (keratinocyte-derived chemokine), MIP-2, granulocyte colony-stimulating factor (G-CSF), IL-1β, monocyte chemoattractant protein-1, IL-12 (p70), and MIP-1α cytokines in the lung tissues of mice subjected to HV_T as compared with SpV control in both genotypes (Figure 3).

However, we found significantly greater levels of IL-6, KC, and G-CSF (Figure 3A) in the lungs of *Nrf2*^{-/-} compared with *Nrf2*^{+/+} mice (Figure 3A). MV-induced levels of IL-12 (p40) were almost 3-fold higher in *Nrf2*^{-/-} mice and were only modestly altered in *Nrf2*^{+/+} mice (Figure 3A). In contrast, IL-1β, monocyte chemoattractant protein-1, MIP-1α, and MIP-2 levels did not differ significantly between the two genotypes (Figure 3B). There were no statistical differences in the levels of IL-3, IL-4, IFN-γ, and IL-12 (p70) cytokines (data not shown) in either genotype in response to MV as compared with their respective SpV control groups. These experiments suggest that elevated levels of IL-6, KC, IL-12 (p40), and G-CSF may play a role in perpetuating VILI in *Nrf2*^{-/-} mice.

MV Induces Redox Imbalance in the Lungs of *Nrf2*^{-/-} Mice

Oxidant and antioxidant (redox) imbalance seems to regulate the pathogenesis of various lung diseases (17). Several studies, including ours, recently demonstrated that exposure to cyclic stretch causes reactive oxygen species (ROS) generation in a magnitude-dependent manner in lung epithelial cells, suggesting a role for redox imbalance in VILI (19–21). Therefore, we examined whether Nrf2 deficiency leads to redox imbalance, thereby resulting in an enhanced susceptibility to VILI. To address this aspect, we first assessed the effects of MV on antioxidant enzyme (mRNA and protein) expression in the lungs of *Nrf2*^{+/+} and

Figure 3. Effect of mechanical ventilation (MV) on cytokine expression in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. Lung tissues of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice subjected to spontaneous ventilation (SpV) or high V_T (HVt), as detailed in Figure 1, were homogenized and centrifuged. Supernatants were collected, and protein was estimated by the BCA method. Equal amounts of protein were used for the cytokine multiplex bead-based immunoassay kit as detailed in METHODS. The concentration of each cytokine in the lung tissues was quantified using a standard curve, and the levels of each cytokine were expressed as pg/mg lung tissue. (A) Cytokine profiles altered by MV that differed between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. (B) Cytokine profiles altered by MV that did not differ between two genotypes. Data are means $\pm$ SEM ($n = 3-4$ mice per group). * $P \leq 0.05$, HVt versus SpV groups of each genotype. ** $P \leq 0.001$, HVt versus SpV groups of the respective genotype. # $P \leq 0.05$, $Nrf2^{-/-}$ mice versus $Nrf2^{+/+}$ mice subjected to HVt.

$Nrf2^{-/-}$ mice subjected to HVt or sham-operated conditions (Figure 4A). Total lung RNA was isolated, and the expression of Gclc, Gclm, and Gpx2 was determined by quantitative real-

time polymerase chain reaction. We chose these antioxidant enzymes because they are well characterized prototypic targets of Nrf2 and play key roles in maintaining cellular redox balance

Figure 4. Effects of mechanical ventilation (MV) on antioxidant gene expression and reduced glutathione/oxidized glutathione (GSH/GSSG) levels. (A) Mice were subjected to spontaneous breathing (sham-treated) or MV at HVt for 2 hours, and total RNA and protein were isolated as detailed in METHODS. cDNA was produced by reverse-transcription of RNA (100 ng) extracted from the left lungs of mice ($n = 3$ per group) followed by polymerase chain reaction amplification using primers specific for mouse Gclc, Gclm, and Gpx2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (B) Western blot analysis of glutathione-S-transferase (GST) isoenzymes in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. (C) Bands were quantified using a Bio-Rad Gel Doc 2000 System. Data are means $\pm$ SEM from two inde-

pendent experiments and are expressed as percentage increases relative to the vehicle-treated $Nrf2^{+/+}$ group. Effect of MV on the specific activities of GST (D) and GPx (E) enzymes in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. Values represent means $\pm$ SEM ($n = 3$ per group). * $P \leq 0.05$, HVt versus control (SpV) of the same genotype. (F) GSH/GSSG ratios of lung tissue samples of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice subjected to SpV and MV at HVt were determined as detailed in METHODS. Data are presented as means $\pm$ SEM ($n = 3-4$). * $P \leq 0.05$, HVt versus SpV control of $Nrf2^{-/-}$ mice.

(24). Constitutive expression of these enzymes in sham-treated *Nrf2*^{-/-} mice was lower than in their counterpart *Nrf2*^{+/+} mice (Figure 4A). MV significantly increased the mRNA expression of Gclc, Gclm, and GPx2 in the *Nrf2*^{+/+} mice over basal levels (Figure 4A). In contrast, the inducible expression of these enzymes in *Nrf2*^{-/-} mice subjected to MV was considerably lower than in their counterpart wild-type animals.

In a separate group of animals, total lung protein was isolated from the lungs of mice subjected to MV or SpV. An equal amount of protein was used for immunoblot analysis with antibodies specific for glutathione-S-transferase (GST) isoenzymes GSTa1, GSTa2, and GSTm1. Constitutive expression of these enzymes was significantly lower in the lungs of *Nrf2*^{-/-} mice than in *Nrf2*^{+/+} mice (Figure 4B). The levels of GSTa1, GSTa2, and GSTm1 were not significantly altered by MV in both genotypes (Figure 4C). The total GST (Figure 4D) and GPx activity (Figure 4E) in the lungs of *Nrf2*^{+/+} mice subjected to air or MV (72 h) was significantly higher (1.6- to ~2.2-fold) compared with *Nrf2*^{-/-} mice. These results suggest that MV induces redox imbalance in the lungs of *Nrf2*^{-/-} mice. To confirm this notion, we analyzed the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) as a measure of the antioxidant imbalance, in lung tissue homogenates of mice subjected to HV_T and sham-operated conditions (Figure 4F). In response to MV stimulus, there was a significant increase in the levels of GSH oxidation (i.e., a decrease of GSH/GSSG ratio) in the lungs of *Nrf2*^{-/-} mice subjected to MV (bar 4) compared with the SpV control group (bar 2) (Figure 4F). In contrast, there was no significant change in GSH/GSSG ratio in wild-type animals subjected to MV.

Antioxidant Supplementation Attenuates VILI in Mice

A decrease in GSH/GSSG ratio and lack of a significant induction of Nrf2-dependent cytoprotective response suggested that redox imbalance may contribute to the greater levels of lung injury and inflammatory response in *Nrf2*^{-/-} mice. To validate this notion, we investigated whether exogenous antioxidant supple-

mentation could provide protection against MV-induced lung injury and inflammation in *Nrf2*^{-/-} mice. *Nrf2*^{-/-} mice were supplemented with NAC 60 minutes before MV exposure. PBS was used as vehicle control. After NAC supplementation, mice were randomly assigned to SpV or MV with HV_T for 2 hours. Lung inflammation and injury were analyzed as detailed previously. MV induced an increase in total number of cells (Figure 5A) and neutrophils (Figure 5B) in the BALF of vehicle-treated groups. However, NAC therapy before MV greatly attenuated MV-induced lung inflammatory responses in *Nrf2*^{-/-} and wild-type animals. There was no significant effect of NAC, as compared with PBS (vehicle), on neutrophil influx in mice subjected to SpV. The dosage of NAC in these experiments, which is three times higher than that used clinically, may have some unwarranted nonspecific effects, such as autooxidation.

We also examined the effect of NAC on MV-induced pulmonary vascular leak as detailed previously. Supplementation of NAC had a significant effect on MV-induced albumin leak in BALF (Figure 5C) and pulmonary extravasation of Evans blue (Figure 5D) in both genotypes as compared with vehicle-treated groups. Taken together, these results strongly support that oxidative stress regulates the pathogenesis of VILI.

Nrf2^{-/-} Mice Subjected to Prolonged MV with LV_T Have Greater Susceptibility than Wild-Type Mice

Our results indicate that *Nrf2*^{-/-} mice subjected to HV_T for 2 hours display greater levels of lung injury and inflammation as compared with wild-type mice when exposed to MV. To determine whether disruption of Nrf2 enhances susceptibility to clinically relevant tidal volumes for longer ventilatory periods, *Nrf2*^{+/+} and *Nrf2*^{-/-} mice were subjected to LV_T and SpV for 4 hours, after which lungs were fixed with formalin and embedded in paraffin or used for collection of BALF for differential cell counts and protein estimation. Compared with genotype-matched SpV controls, a longer period of MV at LV_T caused a statistically significant increase in mean protein accumulation in *Nrf2*^{+/+}

Figure 5. Effects of exogenous antioxidant on mechanical ventilation (MV)-induced lung inflammation and injury. (A and B) Wild-type and *Nrf2*^{-/-} mice were pretreated for 30 minutes with *N*-acetyl-L-cysteine (NAC) (100 mg/kg body weight) before MV. Mice were randomly assigned to spontaneous ventilation (SpV) or high V_T (HV_T) groups. Mice were subjected to MV for 2 hours and then allowed to recover for 1 hour. Bronchoalveolar lavage fluid (BALF) was collected to measure the total cells (A) and neutrophils (B) as detailed previously. Data are presented as means $\pm$ SEM ($n = 4-6$). The albumin concentration in the BALF was analyzed by ELISA. (C) The values of the static control group were considered to be 100%. MV-induced albumin leakage is presented as the percentage increase over static control ($n = 4-6$ animals/group). * $P < 0.05$ versus vehicle control. (D) The effect of NAC intervention on MV-induced vascular leakage was assessed by extravasation of Evans blue into the lung parenchyma. *Nrf2*^{+/+} and *Nrf2*^{-/-} mice were pretreated with NAC as described previously and subjected to MV at HV_T or SpV

for 2 hours. Evans blue was injected into the jugular vein 30 minutes before termination of ventilation, and extravasation of Evans blue in the lung tissue was quantified and expressed as $\mu\text{g}/\text{mg}$ lung tissue. Values represent $n = 5$ to 6 mice per experimental condition. The values for the static control group were considered to be 100%. MV-induced albumin leakage in the lung is expressed as the percentage increase over the static control.

* $P \leq 0.05$. EBD = Evans blue dye.

and *Nrf2*^{-/-} mice. However, the amount of protein found in the BALF of *Nrf2*^{-/-} mice subjected to LV_T was higher than that in *Nrf2*^{+/+} mice subjected to identical V_T ($P < 0.06$) (Figure 6A). Differential cell count analysis revealed significantly higher levels of neutrophils in *Nrf2*^{-/-} and *Nrf2*^{+/+} mice subjected to MV at LV_T as compared with respective SpV control groups. We found significantly higher number of neutrophils in the BALF of *Nrf2*^{-/-} mice than *Nrf2*^{+/+} mice (Figure 6B; $P < 0.0001$) after LV_T. Longer periods of MV at LV_T markedly increased total cells

Figure 6. Lung injury and inflammatory response of *Nrf2*^{-/-} and *Nrf2*^{+/+} mice subjected to mechanical ventilation (MV) at low V_T (LV_T) for 4 hours. (A) Mice ($n = 5-6$ mice per group) were subjected to MV at LV_T or spontaneous ventilation (SpV) for 4 hours, and lung injury and neutrophil infiltration were assessed as detailed in Figure 2. Total cells (A), neutrophils (B), macrophages (C), and protein (D) in the bronchoalveolar lavage fluid (BALF) were analyzed as in Figure 1. * $P \leq 0.05$, LV_T versus SpV of *Nrf2*^{+/+} mice. ** $P \leq 0.05$, LV_T versus SpV of *Nrf2*^{-/-} mice. # $P \leq 0.05$, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to LV_T. (E) Neutrophils in the alveolar space were quantified by immunohistochemistry using antineutrophil antibody as in Figure 2B. Data represent means $\pm$ SEM ($n = 5-6$). * $P \leq 0.001$, LV_T versus SpV of *Nrf2*^{+/+} mice. ** $P \leq 0.004$, LV_T versus SpV of *Nrf2*^{-/-} mice. # $P \leq 0.002$, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to LV_T. (F) Vascular leakage was assessed by the extravasation of Evans blue into lung parenchyma. Mice ($n = 6-7$ per group) subjected to SpV or MV at LV_T for 4 hours were injected with Evans blue into the jugular vein 30 minutes before termination. * $P \leq 0.02$, LV_T versus SpV of *Nrf2*^{+/+} mice. ** $P \leq 0.001$, LV_T versus SpV of *Nrf2*^{-/-} mice. # $P \leq 0.05$, *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice subjected to LV_T. EBD = Evans blue dye.

and macrophages in BALF of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice, but no significant differences were noted between *Nrf2*^{+/+} mice and *Nrf2*^{-/-} mice. To assess the effects of MV on neutrophil infiltration, lung tissue sections were stained with antineutrophil antibody, and the number of neutrophils present in the alveolar septa was quantified as in Figure 2B. The analysis revealed no statistically significant difference in the number of neutrophils in both genotypes subjected to SpV (Figure 6E). There was a significant increase in the number of neutrophils present in the lungs of *Nrf2*^{+/+} mice (17 vs. 11 cells; $P < 0.001$) and *Nrf2*^{-/-} mice (22 vs. 14 cells, respectively; $P < 0.004$) subjected to LV_T as compared with the respective SpV control groups. However, *Nrf2*^{-/-} mice had significantly more neutrophils compared with *Nrf2*^{+/+} mice subjected to LV_T (22 vs. 17 cells; $P < 0.002$).

We assessed vascular leakage in a separate group of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice subjected to LV_T for 4 hours, and the results were compared with those of respective SpV control groups. MV at LV_T for 4 hours caused a significant increase in extravasation of Evans blue in both genotypes compared with their respective SpV control groups (Figure 6F). However, exposure to LV_T caused greater accumulation of Evans blue in lungs of *Nrf2*^{-/-} mice compared with *Nrf2*^{+/+} mice ($P < 0.05$). There was no significant difference between these genotypes exposed to SpV. Taken together, these results demonstrate that a longer period (4 h) of MV at LV_T, in contrast to a shorter period (2 h), induces lung injury and inflammation and that the lack of Nrf2 transcriptional response further enhances the severity of VILI.

Expression Profiling Genes Regulated by Nrf2 in Response to MV

To further define the mechanisms by which Nrf2 modulates MV-induced inflammatory responses, global gene expression profiles were examined in the lungs of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice subjected to MV at HV_T or SpV for 2 hours. The full list of genes that were up- or down-regulated by MV in the lungs of *Nrf2*^{+/+} and *Nrf2*^{-/-} mice (see Tables E1-E4 in the on-line supplement) and validation real time polymerase chain reaction data for some of these genes (Figure E1) is presented in the online supplement. Expression profiling revealed 197 up-regulated (Table E1) and 70 down-regulated genes (Table E2) in the lungs of *Nrf2*^{+/+} mice and 193 up-regulated (Table E3) and 85 down-regulated genes (Table E4) in the lungs of *Nrf2*^{-/-} mice after MV. These genes were further analyzed to dissect out the Nrf2-regulated transcriptional programs and the differences in the expression profiles between *Nrf2*^{+/+} and *Nrf2*^{-/-} mice that are known to be altered by MV and associated with ventilator-induced lung injury/ventilator-associated lung injury (VILI/VALI), as shown in Table 1. The expression profiles of genes encoding several redox regulators, stress response proteins, transcription factors, kinases, phosphatases, and solute transporter proteins are also shown in Table 1.

VALI genes. Our results, consistent with previous reports (11, 15, 33-35), demonstrated an inducible expression of a majority of VALI genes in wild-type (*Nrf2*^{+/+}) mice (Table 1); however, we found a lack of induction of several of these genes in the lungs of *Nrf2*^{-/-} mice. For example, several genes that modulate tissue matrix remodeling and blood coagulation were significantly induced in *Nrf2*^{+/+} mice but not in *Nrf2*^{-/-} mice. These genes include Adamts1, Thbs1, Plaur, coagulation factor III (F3), Cyr61, and proteinase inhibitors Serpine 1 and Timp3. MV markedly induced the expression of Ptgs2 (Cox2), but no significant difference in the inducible levels of this enzyme between the two genotypes was observed. Although MV significantly induced the levels of amphiregulin, an EGFR ligand, in both genotypes, the induction was markedly lower in *Nrf2*^{-/-} mice as compared with

Nrf2^{+/+} mice. VALI genes that regulate lung inflammation, such as *Tnfr12a*, *IL-6*, *Cxcl1*, and *Socs3*, were elevated by MV in *Nrf2*^{+/+} mice but not in *Nrf2*^{-/-} mice. The mRNA levels of these genes were not altered by MV in *Nrf2*^{-/-} mice. On the contrary, we found that *Il-1β* and *Cxcl2* were down-regulated by MV in *Nrf2*^{-/-} mice. We also found diminished levels of *Tnfrsf19* and *Traf1* in *Nrf2*^{+/+} mice in response to MV. Members of the IFN family were distinctly altered by MV in wild-type and *Nrf2*^{-/-} mice. MV strongly induced expression of IFN-related developmental regulator 1 (*Irf1*), whereas that of IFN-induced protein with tetratricopeptide repeats 2 (*Ifit2*) and IFN-inducible GTPase 1 (*Iigp1*) was down-regulated in wild-type mice. In contrast, we found elevated levels of IFN-activated gene 203 (*Ifit203*) in *Nrf2*^{-/-} mice exposed to MV but not in *Nrf2*^{+/+} mice.

Transcription factors. Several genes that regulate transcription, such as *Atf3*, *Cebpd*, *Egr1*, *Egr2*, *Fos*, *Gadd 45γ*, *Nr4a1*, *Nr4a2*, *Srf*, and *Mafk*, were significantly induced in *Nrf2*^{+/+} mice, but their induction is very low or not induced significantly above basal levels in the lungs of *Nrf2*^{-/-} mice. The inducible levels of *Ppargc1α* expression did not differ significantly between *Nrf2*^{+/+} and *Nrf2*^{-/-} mice. MV significantly induces the expression of several other transcription factors that have not been directly linked to VILI. The expression levels of *Fosb*, *Klf6*, *Klf7*, *Trib1*, and *Smad1* were significantly induced in *Nrf2*^{+/+} but not in *Nrf2*^{-/-} mice. In contrast, MV down-regulated the expression levels of *Sox7*, *Sox18*, and *Stat5b* levels in *Nrf2*^{+/+} mice, but these levels were unchanged in *Nrf2*^{-/-} mice.

Stress response genes. *Nrf2* regulates the expression of several antioxidant genes. We found markedly lower expression of *Gst* family members in the lungs of *Nrf2*^{-/-} mice than in *Nrf2*^{+/+} mice in SpV control groups. The expression of these genes was not altered significantly in *Nrf2*^{+/+} mice after MV challenge. However, the level of *Gclc* and *Txrdn1* expression was significantly higher in *Nrf2*^{+/+} mice than in *Nrf2*^{-/-} mice after MV exposure. The expression levels of members of the NADPH oxidase family, such as neutrophil cytosolic factor-2 (*Ncf2* or p67-phox), neutrophil cytosolic factor-4 (*Ncf4* or p47-phox), and *Nox4* (NADPH oxidase), were significantly lower in the lungs of *Nrf2*^{-/-} mice after MV. The expression levels of these genes were unchanged in the lungs of *Nrf2*^{+/+} mice in response to MV. Metallothionein-1 and -2 gene expression was markedly up-regulated by MV in *Nrf2*^{+/+} but not in *Nrf2*^{-/-} mice.

We found down-regulation of expression of several heat shock protein (Hsp) family members in the lungs of *Nrf2*^{-/-} mice after MV. These genes include *Hspa1a* (Hsp70 kD protein 1a), *Hspa1b* (Hsp70 kD protein 1b), *Hspa8* (Hsp70 kD protein 8), *Dna1a1* (Hsp40 kD protein 4), and *Dna1b1* (Hsp40 kD protein 4). *Carhsp1* (calcium-regulated heat-stable protein of 24 kD) was markedly down-regulated in the lungs of *Nrf2*^{-/-} mice. In contrast, MV down-regulated expression levels of *Hsp105* and *Hspa12b* in *Nrf2*^{+/+} mice but not in *Nrf2*^{-/-} mice.

Signal transducers. Kinases and phosphatases play key roles in the modulation of signal transduction induced by external stimuli, such as MV. These genes were differentially expressed between wild-type and *Nrf2*^{-/-} mice after MV (Table 1). Dual-specificity phosphate (*Dusp*)-1, *Dusp8*, and *Dusp16* were up-regulated by MV only in wild-type mice. Likewise, the expression of protein tyrosine phosphatase 4a1 (*Ptp4a1*) and protein tyrosine phosphatase, nonreceptor type 2 (*Ptpn2*) was significantly induced in *Nrf2*^{+/+} mice. The mRNA expression of various kinases, such as hexokinase (*Hk2*), pyruvate dehydrogenase kinase isoenzyme 4, and receptor-interacting serine threonine kinase 4, and a nonreceptor tyrosine kinase *BMX*, was elevated in *Nrf2*^{+/+} but not in *Nrf2*^{-/-} mice. *Ptp4a1* and protein phosphatase 2 regulatory subunit B (PR 52), alpha isoform (*Ppp2r2a*), were only induced by MV in the lungs of *Nrf2*^{-/-} mice.

Solute transporters. Profiling experiments revealed differential expression of several members of the solute carrier (Slc) family of proteins between the lungs of wild-type and *Nrf2*^{-/-} mice (Table 1). Genes encoding for Slc family 23 member 2 (*Slc23a2*) (nucleobase transporter), Slc family 2 member 1 (*Slc2a1*, facilitated glucose transporter), Slc family 20 member 1 (*Slc20a1*, sodium/hydrogen exchanger), Slc family 7 member 5 (*Slc7a5*, cationic amino acid transporter), and Slc family 38 members 2 (*Slc38 a2*) and 4 (*Slc38a4*) were up-regulated by MV in wild-type animals. The expression levels of these genes, except *Slc38 a2*, were not altered significantly in *Nrf2*^{-/-} mice. However, *slc25a4* expression was elevated in *Nrf2*^{-/-} mice by MV as compared with the SpV control group. *Slc4a1* expression was down-regulated by MV in the lungs of both genotypes.

DISCUSSION

The present study clearly establishes that disruption of the oxidative stress modifier *Nrf2* exacerbates MV-induced lung permeability and inflammation (i.e., neutrophil accumulation) and is accompanied by greater levels of several proinflammatory cytokines in lung tissue. These changes were associated with a lack of induction of critical antioxidant enzymes essential for GSH biosynthesis and redox imbalance in *Nrf2*^{-/-} mice after MV. Antioxidant intervention significantly reduced lung alveolar and vascular permeability as well as inflammatory responses in *Nrf2*^{-/-} mice. Collectively, our data demonstrate for the first time that *Nrf2*-dependent antioxidant transcriptional responses act in concert to counteract MV-induced oxidative stress, which otherwise contributes to the pathogenesis of VILI.

The loss of airway epithelial and endothelial integrity resulting in enhanced levels of pulmonary edema is a hallmark of ALI and ARDS (36). Similarly, the influx of protein leak in the lungs of mice exposed to MV and various oxidants (hyperoxia or acid aspiration) and toxins (endotoxin) is well documented in various experimental models of ALI/ARDS (1). In our studies, lung vascular permeability was significantly enhanced in *Nrf2*^{-/-} mice as compared with *Nrf2*^{+/+} mice after MV. Consistent with these results, MV-induced albumin leakage was markedly higher in *Nrf2*^{-/-} mice than *Nrf2*^{+/+} mice. We did not find significant lung injury, as assessed by alveolar protein or albumin content in the BALF (see Figures 1A and 1B), in *Nrf2*^{-/-} mice subjected to MV at LV_T in our acute (2-h) experimental model of VILI, although we noticed greater levels of neutrophils in *Nrf2*^{-/-} mice as compared with SpV control mice. This result suggests that neutrophil accumulation is not dependent on lung epithelial injury in our acute model of VILI. This phenomenon has been observed in various models of ALI by other laboratories (37–39). Our experiments revealed that MV at LV_T for longer periods (4 h) produced significant lung injury and vascular leak, which was associated with elevated levels of lung neutrophil infiltration in wild-type and *Nrf2*^{-/-} mice (Figure 6).

Inflammatory response induced by injurious MV contributes to VILI (8). We found that MV causes a greater increase in the total number of neutrophils in BALF and in lung alveolar septa of *Nrf2*^{-/-} mice than *Nrf2*^{+/+} mice, suggesting that *Nrf2*-dependent transcriptional response may limit inflammatory responses induced by MV. The accumulation of neutrophils in the lung tissue can lead to enhanced levels of inflammatory cytokines, which play fundamental roles in the development of lung pathogenesis, including VALI (9, 10). Multiplex cytokine assays revealed significantly greater levels of KC (human homolog of CXCL1), *IL-6*, and *G-CSF* concentrations in the lungs of *Nrf2*^{-/-} mice compared with *Nrf2*^{+/+} mice (see Figure 3A). KC and MIP-2 chemokines are potent neutrophil chemoattractants and play

a critical role in lung inflammatory responses induced by MV (8). Several studies have shown that exposure to MV or cyclic stretch increases the expression levels of KC (40–42). Treatment of mice with antibodies specific for KC or disruption of CXCR2, the KC receptor, markedly attenuate neutrophil sequestration and lung injury in a murine model of VILI suggesting that KC-CXCR2 activated signaling regulates pathogenesis in this syndrome (42). Elevated levels of KC in the lungs of *Nrf2*^{-/-} mice are consistent with these studies and suggest that *Nrf2* deficiency enhances the expression levels of KC, thereby resulting in enhanced neutrophil accumulation and lung injury. We also found enhanced levels of G-CSF, which is important for neutrophil accumulation and activation in the lungs (43). High levels of G-CSF correlate with ARDS in humans (44–46), and administration of G-CSF induces ALI in animals (43, 47). We also found elevated levels of IL-6 in the lungs of *Nrf2*^{-/-} mice compared with *Nrf2*^{+/+} mice in response to MV, suggesting that this cytokine may play a role in causing VILI. Consistent with this notion, elevated levels of IL-6 have been detected in various experimental models of VILI (48, 49) and in BALF of patients with ARDS on MV (2, 50). Further studies are warranted to dissect temporal induction of these proinflammatory cytokines and their contribution to the development or susceptibility to VILI in *Nrf2*^{-/-} mice in our experimental conditions. The mechanisms by which *Nrf2* deficiency enhances the expression levels of KC, G-CSF, and IL-6 cytokines in response to MV are unclear. However, we have recently shown that disruption of *Nrf2* potentiates nuclear factor (NF)- κ B activation in a murine model of sepsis (28). NF- κ B is critical for the induction of various cytokines in several experimental models of lung injury. MV has been shown to activate NF- κ B and other transcription factors, such as AP-1 (41). Whether *Nrf2* directly or indirectly, through the modulation of NF- κ B, AP-1, and/or other factors, regulates the expression levels of proinflammatory cytokines in response to MV warrants further study.

GCLC and GCLM are essential for the biosynthesis of GSH, a major cellular antioxidant. GPx2 scavenges hydrogen peroxide and organic hydroperoxides. These enzymes play key roles in the maintenance of intracellular redox balance (24). Our experiments revealed (1) failure to increase levels of Gclc, Gclm, and Gpx2; (2) reduced levels of GST and Gpx2 enzyme activities; and (3) greater redox imbalance (i.e., decreased in GSH/GSSG ratio) in *Nrf2*^{-/-} mice compared with wild-type mice in response to MV. In contrast, MV stimulates the expression of Gclc, Gclm, and Gpx2 without a significant effect on the lung redox status in wild-type animals, which are less susceptible to VILI than their *Nrf2*^{-/-} counterparts. We have previously reported that C57BL/6 mice subjected to MV at a V_T of 20 ml/kg for 2 hours demonstrate significant lung injury and inflammation when compared with animals exposed to V_T at 7 ml/kg or sham-operated controls (31, 32). However, we found that the CD1/ICR strain of mice (wild-type and *Nrf2*^{-/-}) were more resistant to HVT in terms of developing VILI (as assessed by protein leakage in BALF), as compared with C57BL/6, and thus required larger V_T to cause injury. Consistent with these results, we have previously reported that CD1/ICR mice have intrinsic resistance to oxidative stress-mediated emphysema compared with C57BL/6 mice (27). This resistance was mainly attributed to high-level induction of antioxidant genes in CD1/ICR mice compared with C57BL/6J mice in response to prooxidants, such as cigarette smoke (51). CD1/ICR mice, when subjected to a longer ventilatory period of MV at LV_T, display significant lung injury and inflammation.

Neutrophil accumulation in lung tissue can lead to enhanced levels of ROS, and antioxidant enzymes are essential in the response to ROS generated by neutrophils (52). Recently, we have

shown that *Nrf2* deficiency diminishes the levels of several antioxidant enzymes and enhances levels of ROS in neutrophils in response to LPS (53). We and others have also shown that exposure to pathologically relevant cyclic stretch leads to high levels of ROS and redox imbalance in lung epithelial cells *in vitro* (20, 21). Thus, it is likely that ROS generated by pulmonary cell types in response to MV may contribute to redox imbalance in *Nrf2*^{-/-} mice. Expression profiling from various laboratories (including ours) demonstrated that *Nrf2* regulates the expression of several genes, in addition to antioxidant enzymes (24). However, the present study demonstrates that antioxidant intervention remarkably attenuates MV-induced lung vascular injury and neutrophil accumulation in *Nrf2*^{-/-} mice, suggesting that oxidative stress mainly contributes to VILI, which is mitigated by an antioxidant response. In preliminary results, Syrika and colleagues showed protective effects of NAC against MV-induced neutrophil influx in a rat model of VILI (54). Recently, we have shown that specific inhibition of xanthine oxidoreductase activity, which is required for ROS generation, attenuates MV-induced lung injury and inflammation (32), further underscoring a role for oxidative stress in the development and/or perpetuation of lung injury.

Expression profiling demonstrated that disruption of *Nrf2* diminishes the inducible expression of several VILI genes (Table 1) that are known to be induced by MV in different experimental models of VILI (11, 15, 33–35). The induction of several genes that modulate tissue matrix remodeling and blood coagulation in *Nrf2*^{+/-} mice but not in *Nrf2*^{-/-} mice strongly suggests that the *Nrf2* transcription factor is critical for up-regulating gene expression in response to MV (Table 1). There was no significant difference in the expression of proinflammatory genes between the genotypes, with the exception of IL-6 and Cxcl1 (up-regulated in *Nrf2*^{+/-} mice) and Il-1 β and Cxcl2 (down-regulated in *Nrf2*^{-/-} mice). Ptg2 (Cox2) regulates lung inflammation; however, we found no significant differences in the expression levels of this enzyme between the two genotypes subjected to MV. Our results suggest that dysregulation of cytokine and tissue matrix remodeling proteins may play a role in enhancing the susceptibility of *Nrf2*^{-/-} mice to VILI. In contrast to mRNA expression profiling, we found greater levels of proinflammatory cytokines, such as KC, IL-6, and G-CSF (measured by multiplex cytokine assays), in the lungs of *Nrf2*^{-/-} mice (see Figure 3A). Cytokine assays and microarray experiments were performed in lung tissues harvested at the termination of MV at 2 hours. It is well known that various cytokines are induced, at the transcriptional level, within 30 to 60 minutes in response to various stimuli in lung tissues/cell types. For example, we recently reported a robust induction of proinflammatory genes, such as IL-6, MIP-1 α (Ccl3), MIP-1 β (Ccl4), MIP-2 (IL-8), and KC (Cxcl or Gro- α), in the lungs of *Nrf2*^{-/-} mice after 30 minutes of LPS administration, as compared with wild-type mice (28). Thus, the observed discordance between mRNA and protein expression profiling may be at least in part attributable to temporal induction of patterns of cytokines. Elaborative lung mRNA and protein expression profiling studies of *Nrf2*^{+/-} and *Nrf2*^{-/-} mice subjected to MV at LV_T and HVT, performed at earlier time points (30–60 min), are warranted to further decipher *Nrf2*-regulated inflammatory cytokine and chemokine transcriptional programs.

Nrf2 deficiency affects the induction of several antioxidant genes. Although a lack of induction of antioxidant genes was anticipated, expression profiling revealed that deficiency of *Nrf2* results in down-regulation of several HSPs after MV. HSPs, by acting as molecular chaperones, limit protein aggregation and facilitate protein folding and transport of other proteins, thereby providing cytoprotection and maintenance of cellular homeostasis. The down-regulation of the HSP family members Hspa1a,

Hspa1b, and Hspa8 by MV in *Nrf2*^{-/-} mice but not in wild-type mice suggests that the Nrf2 transcriptional response is critical for the maintenance of HSP levels in the lung after mechanical stimuli. In support of this notion, Weiss and colleagues demonstrated that overexpression of Hsp70 in pulmonary epithelium reduces mortality and lung injury in a model of cecal ligation and puncture (55). Hsp70 induction was also associated with decreased pulmonary fibrosis (56), whereas ablation of the heat shock factor-1, which regulates HSP family members expression, increases susceptibility to hyperoxia in experimental models of ALI in mice (57). DnaJ (Hsp40) family proteins, DnaJ1 and DnaJb1, which are down-regulated by MV in the absence of Nrf2, by acting as co-chaperones modulate Hsp70 activity (58). Carhsp1, a calcium-regulated, heat-stable protein, is involved in calcium-mediated signal transduction (59). The expression of this gene is also markedly lower in *Nrf2*^{-/-} mice subjected to MV. Thus, it is likely that Nrf2 up-regulates the expression of several HSP family members to maintain cellular homeostasis in response to MV.

The microarray results suggest that Nrf2 regulates signal transduction pathways by controlling the expression of several transcription factors, kinases, and phosphatases. Some of these proteins have been linked to VILI. We found striking differences in the expression levels of VALI genes encoding ATF3, Gadd45γ, Fos, EGR1, and Nr4a1 transcription factors. Up-regulation of these transcripts was found by various laboratories in different experimental models of VILI (11, 14, 15, 33–35). Gadd45γ and ATF3 transcription factors are known to regulate gene expression involved in cellular stress, whereas EGR1 and Fos are immediate early response genes important for the expression of various cytokines and growth factors. We found that Nrf2 is critical for the induction of Klf6, Klf7, and Trib1 by MV. Trib1 differentially modulates the duration and the specificity of MAP kinase activation (60, 61). We found a marked induction of Trib1 in wild-type mice but not in *Nrf2*^{-/-} mice, suggesting that Nrf2 response modulates MV-induced signaling in the lung. Consistent with this result, various genes encoding protein phosphatases, such as Dusp1, Dusp8, Dusp16, Ptp4a1, and Ptpn2, are induced by MV in the presence of Nrf2 but not in its absence. Previous studies have demonstrated that inhibition of P13K-Akt signaling and JNK1/2 kinases attenuates VILI in mice (62, 63). Although the exact roles of these protein phosphatases in the development of lung pathogenesis induced by MV and other stressful stimuli remain unclear, it is likely that these proteins may play a role in modulating the duration and/or magnitude of the activity of various kinases induced by MV.

We found striking differences in the expression levels of various Slc proteins in the lungs of wild-type and *Nrf2*^{-/-} mice subjected to MV. There are at least 108 Slc proteins reported that are selectively expressed in various tissues, including the lung (64). These proteins play key roles in the transport of various substrates, such as saccharides, amino acids, ions, metals, and peptides. The differential induction of Slc2a1 (facilitated glucose transporter), Slc7a5 (L-type amino acid transporter), Slc20a1 (phosphate transporter), Slc23a2 (vitamin C transporter), Slc25a4 (adenine nucleotide translocator), and Slc38a2 and Slc38a4 (amino acid transporters) suggests that Nrf2 deficiency may result in a dysregulated absorption and distribution of various substrates, thereby enhancing the severity of VILI. NCF2 and NCF4 are critical for activation of the NOX4 (NADPH oxidase), which transfers electrons from NADPH to molecular oxygen, thereby resulting in the production of reactive superoxide. Paradoxically, we found that NCF2 and NCF4 are down-regulated in the lungs of *Nrf2*^{-/-} mice in response to MV, raising the possibility that a lack of Nrf2 may result in activation of compensatory mechanisms that suppress the expression of these enzymes, probably

to dampen high levels of superoxide generation. Although the majority of VALI genes are induced by MV wild-type mice (CD1/ICR strain) in our experimental conditions, we found certain discrepancies in the expression levels of several genes. For example, in our experiments we found the induction of GADD45γ, instead of the GADD45α isoform as reported by others (34, 35). We attribute this and other variations to differences in the genetic background of mice or slight variations in experimental conditions related to MV.

In summary, our data suggest that maintenance of a proper redox balance regulated by an Nrf2-dependent transcriptional program might be essential for the integrity and function of different lung cellular components and the outcome of VILI. We found functional polymorphisms in the promoter region of human NRF2, which were associated with an increased risk of ALI in a population of patients with major trauma (65), further underscoring a critical role for Nrf2-dependent signaling in ALI/ARDS. We therefore speculate that dysregulation or variation in Nrf2 signaling pathway(s) may enhance susceptibility to VILI due to lack of a proper redox balance in the lungs. Consistent with this notion, expression profiling studies revealed that Nrf2 regulates, in addition to VALI genes, several novel networks, such as stress proteins, transcription factors, phosphatases, and solute carrier proteins. Analyzing the functional roles of candidate target genes and signal transduction pathways regulated by Nrf2 in response to MV may provide additional insight into the factors that promote or perpetuate VILI and help in developing novel therapies targeted at oxidative stress and inflammation in this syndrome.

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